Very Long Chain Fatty Acids in Higher Animals—A Review

Alf Poulos*

Department of Chemical Pathology, Women's and Children's Hospital, North Adelaide, South Australia, 5006

ABSTRACT: Fatty acids with greater than 22 carbon atoms (very long chain fatty acids, VLCFA) are present in small amounts in most animal tissues. Saturated and monoenoic VLCFA are major components of brain, while the polyenoic VLCFA occur in significant amounts in certain specialized animal tissues such at retina and spermatozoa. Biosynthesis of VLCFA occurs by carbon chain elongation of shorter chain fatty acid precursors while β-oxidation takes place almost exclusively in peroxisomes. Mitochondria are unable to oxidize VLCFA because they lack a specific VLCFA coenzyme A synthetase, the first enzyme in the β-oxidation pathway. VLCFA accumulate in the tissues of patients with inherited abnormalities in peroxisomal assembly, and also in individuals with defects in enzymes catalyzing individual reactions along the β-oxidation pathway. It is believed that the accumulation of VLCFA in patient tissues contributes to the severe pathological changes which are a feature of these conditions. However, little is known of the role of VLCFA in normal cellular processes, and of the molecular basis for their contribution to the disease process. The present review provides an outline of the current knowledge of VLCFA including their biosynthesis, degradation, possible function and involvement in human disease.
Lipids 30, 1–14 (1995).

Interest in very long chain fatty acids (i.e., fatty acids with greater than 22 carbon atoms, VLCFA) has increased greatly over the last few years since the recognition that this group of fatty acids is associated with certain disorders of peroxisomal structure and/or function. Indeed, the measurement of plasma and tissue VLCFA levels has formed the basis for a biochemical test for peroxisomal function. These studies have confirmed that VLCFA are components of most human tissues, indicating that they play a physiological role in many different cell types. More recent reports of an involvement of VLCFA in the biosynthesis of polyunsaturated fatty acids have stimulated further interest. In this review it is proposed to focus on the VLCFA in higher animals. Readers are referred to earlier reviews on VLCFA in animals and plants, and the involvement of VLCFA in human disease (1,2).

*Address correspondence at Department of Chemical Pathology, Women's and Children's Hospital, 72 King William Road, North Adelaide, South Australia, 5006, Australia.

Abbreviations: ABC, ATP-binding cassette; ALD, adrenoleukodystrophy; ATP, adenosine triphosphate; CoA, coenzyme A; FABP, fatty acid binding protein; TCA, tricarboxylic acid; TNFα, tumor necrosis factor α; VLCFA, very long chain fatty acids.

STRUCTURE AND OCCURRENCE OF VLCFA

Like the shorter chain fatty acids, VLCFA which occur in nature can be divided broadly into three different classes—saturated, monoenoic and polyenoic. These may be subdivided further into hydroxylated and nonhydroxylated, and branched and unbranched chain derivatives.

Saturated and monoenoic VLCFA. The richest source of saturated and monoenoic VLCFA in most higher animals is the brain, and particularly myelin. The brain contains high amounts of 24 and 26 carbon saturated and monoenoic fatty acids and significant amounts of odd-chain 23, 25 and 27 carbon fatty acids. Smaller amounts of fatty acids with 28 and 30 carbon atoms are also found in brain. The monoenoic fatty acids in brain are *cis* configuration and contain predominantly two isomeric forms, n-7 and n-9 homologues (3,4). The relative proportions of the two monoenoic homologues vary quite significantly with carbon chain length. It is interesting to note that the shorter chain, odd-numbered monoenoic fatty acids (i.e., 17, 19 and 21 carbon fatty acids) comprise a mixture of n-8 and n-10 homologues, indicating that they are not precursors for the monoenoic VLCFA. The concentration of VLCFA in the brain increases with development (5,6). In addition to the developmental changes, there is an increase in VLCFA with aging (7). Significant levels of a wide variety of saturated and monoenoic VLCFA also occur in meibomian glands (and their secretions) of cattle, rabbits, mice, rats and humans (8–12). VLCFA comprise up to 23% of the fatty acids of human meibomian glands and their secretions (8). Odd- and even-numbered fatty acids with up to 32 carbon atoms were reported in these glands. Of particular interest is the presence of appreciable amounts of branched chain iso- and anteiso-fatty acids (13). Saturated and monenoic VLCFA occur in small amounts in most tissues and in body fluids. They are trace components of human plasma (14), the two principal plasma VLCFA (24:0 and 24:1) normally constituting less than 1.0%, while 26:0 and 26:1 make up less than 0.1% of the total fatty acid complement. In general, the VLCFA with >26 carbon atoms are not detected in tissues other than brain, skin or in certain glands. However, labeled VLCFA can be detected when cells are incubated with high specific activity [^{14}C]acetate (15), so it is likely that they occur in most tissues.

Saturated and monoenoic fatty acids occur as α-hydroxy derivatives in kidney, stomach (16,17) and, particularly, in

Copyright © 1995 by AOCS Press

mammalian brain (6). α-Hydroxy VLCFA (mainly 24:0, 25:0, 26:0 and 27:0 and their corresponding monenoic derivatives) occur in large amounts in brain, and ω-hydroxy monenoic VLCFA with up to 34 carbon atoms are found in epidermal lipids (18,19).

Only a small proportion of the saturated and monenoic VLCFA occurs unesterified. However, it is difficult to know whether the small amounts of unesterified VLCFA found, for instance in tissue such as brain (Poulos, A., and Sharp, P., unpublished observations), are simply the result of post-mortem changes. Depending on the tissue, VLCFA are joined either in ester or amide linkage, in a number of different lipid species including cholesterol esters, gangliosides, galactocerebroside, sphingomyelin, and phosphatidylcholine (20–23). α-Hydroxy VLCFA are bound almost exclusively in amide linkage in cerebroside sulfate and galactocerebroside (6,24), while ω-hydroxy VLCFA are bound to unusual acylglucosyl ceramide and acylceramide species (19,25). More recent reports indicate that significant amounts of saturated and monoenoic VLCFA are bound in thioester linkage to myelin proteolipid protein (26–28). Covalently-bound lipids containing VLCFA are also found in human stratum corneum lipids (29,30).

Unsaturated VLCFA. The occurrence of polyenoic VLCFA (mainly 24:4n-6 and 24:5n-6) in rat testis was reported over twenty years ago (31,32). Later studies confirmed the presence of a homologous series of n-6 polyenoic VLCFA with up to 30 carbon atoms in rat and mouse testis (33–35). More recently, polyenoic VLCFA have been detected in mature spermatozoa of a number of mammalian species including man (36,37), in rat epididymal spermatozoa (38) and in rat seminiferous tubules (39). Mammalian spermatozoa contain polyenoic VLCFA with up to 34 carbon atoms and from 3–6 methylene-interrupted double bonds. In some species the polyenoic VLCFA comprise up to 5% of the total fatty acids. Both n-3 and n-6 fatty acids have been detected in spermatozoa, although in individual species there is a predominance of one series or the other. Ram and bull spermatozoa, which contain significant amounts of both n-3 and n-6 series acids, are particularly rich in the longer chain polyenoic VLCFA, i.e., 30–34 carbon n-3 series hexaenoic acids. The n-6 series fatty acids with 3–5 double bonds predominate in human, boar and rat spermatozoa, while boar and rat contain unusual fatty acids with an odd number of carbon atoms (36,39). The testis and spermatozoa of the latter two species also contain unusual 2-hydroxylated n-6 tetra- and pentaenoic fatty acids with very long carbon chains (40). These fatty acids are joined in amide linkage to sphingomyelin in ram spermatozoa (37). However, in the rat, polyenoic VLCFA are also found esterified in triacylglycerols and cholesterol esters, as well as in sphingomyelin (39). Alkyl diacylglycerols, which are only minor components of rat testis, are particularly rich in polyenoic VLCFA. Around 34% of the total alkyl diacylglycerols fatty acids are fatty acids with carbon chain lengths >22 (39). Rat spermatozoa are also unusual in that they contain appreciable amounts of n-9 series polyenoic fatty acids, including 22:4n-9 and a novel VLCFA, 24:4n-9. These fatty acids appear to be concentrated in choline plasmalogen (41).

Another particularly rich source of polyenoic VLCFA is the retina. The occurrence of polyenoic VLCFA in the retina of diverse vertebrates (fish, birds and mammals) was first reported in the late 1980s (42–45). These fatty acids contain up to 36 carbon atoms and from 4–6 methylene-interrupted *cis* double bonds, and they comprise around 2% of the total fatty acids of retina and up to 13% of the rod outer segments (42). Representatives of both n-3 and n-6 series are present. As observed with testis and spermatozoa, the longer chain fatty acids (30–36 carbon atoms) predominate. Much of the polyenoic fatty acids are present in dipolyunsaturated molecular species of phosphatidylcholine. For example, VLCFA-containing phosphatidylcholines of bovine retina include 30:4n-6/22:6n-3, 32:4n-6/22:6n-3, 34:4n-6/22:6n-3, 30:5n-3/22:6n-3, 32:5n-3/22:6n-3, 34:5n-3/22:6n-3, 30:6n-3/22:6n-3, 32:6n-3/22:6n-3, 34:6n-3/22:6n-3 (43,45), highly hydrophobic species with up to 56 carbon atoms (not including the glycerol moiety) and with as many as 12 olefinic centers. The VLCFA are esterified almost exclusively in the *sn*-1 position of the glycerol molecule (44). The structure of some of these molecular species has been confirmed by fast atom bombardment mass spectrometry (45).

Polyenoic VLCFA were first reported in human brain in patients with the rare inherited disorder, Zellweger's syndrome (46,47). However, later studies demonstrated the presence of VLCFA with up to 38 carbon atoms and from 3–6 methylene-interrupted double bonds in normal young human brain (20,45). The longer chain fatty acids (30–38 carbon atoms) are largely, but not exclusively, tetra- and pentaenoic n-6 series acids, with 34:4n-6 and 34:5n-6 predominating. The polyenoic VLCFA are also components of rat brain. Although 24:4n-6 and 24:6n-3 are the major VLCFA in adult rat brain, there are significant amounts of 34:4n-6 and 36:4n-6 (48). However, there are marked changes in both the concentration and composition of the polyenoic VLCFA with development in rat and human brain. The content of these fatty acids declines with increasing age in the rat. Concomitantly, there is a marked reduction in the levels of pentaenoic VLCFA with age and an increase in the proportion of longer chain VLCFA. For example, 34:4n-6 and 34:5n-6 are the major longer chain polyenoic VLCFA in neonatal rat brain, and 36:4n-6 predominates in adult brain. A similar change with development is observed in human brain (20). Thus n-6 pentaenoic VLCFA predominate in the fetus and in neonates, while the n-6 series tetraenoic acids are major components in older children.

As in the retina, most of these fatty acids are found esterified to the *sn*-1-position of the glycerol moiety in phosphatidylcholine (2,20,45). However, brain differs from retina in that the fatty acids esterified to the *sn*-2 position include arachidonic, oleic and palmitoleic acids. These molecular species are similarly hydrophobic with up to 58 carbon atoms (excluding glycerol) and nine olefinic centers. Whereas the longer chain VLCFA (>32 carbon atoms) are concentrated al-

most exclusively in phosphatidylcholine, those with <32 carbon atoms are also found in the unesterified fatty acid and cholesterol ester fractions (49).

Trace amounts of 24 to 28 carbon tetraenoic n-6 fatty acids (<0.1%) have been reported in human neutrophils (50), and 24:4n-6 is present in human platelets (Robinson, B.S., Ferrante, A., and Poulos, A., unpublished data) and human plasma (51). Studies using labeled fatty acid precursors indicate that 24:4n-6, 24:5n-6, 24:5n-3 and 24:6n-3 are normal components of liver (52), retinoblastoma, fibroblasts (53,54) and cultured endothelial cells (55). It is likely that polyenoic VLCFA are normal components of most animal cells, but sensitive analytical procedures may be required to detect them in some tissues.

BIOSYNTHESIS

Saturated and monenoic VLCFA. Three different pathways are involved in the biosynthesis of saturated fatty acids; a soluble cytoplasmic system which generates fatty acids, mostly palmitic acid, from acetyl coenzyme A (CoA) *via* malonyl CoA, and mitochondrial and microsomal systems. The latter system also uses malonyl CoA as donor while mitochondrial fatty acid synthesis requires acetyl CoA (56,57). It is believed that the microsomal and mitochondrial elongation pathways generate fatty acids with carbon chain lengths >16. Early reports indicated that there were three microsomal pathways involving the elongation of palmitoyl CoA, stearoyl CoA and arachidoyl CoA (16:0, 18:0 and 20:0, respectively) in mouse brain (58), and this has received some recent support (59,60). Inhibitor studies indicate that different processes may be involved in the elongation of 20:0 CoA to 22:0 CoA, and 20:0 CoA to 24:0 CoA in pig neutrophils (60). One possible explanation for these findings is that there may be a specific enzyme system required for the generation of saturated VLCFA from 20:0.

Biosynthesis of monenoic VLCFA proceeds *via* the same elongation pathways except, of course, the precursor fatty acids are the corresponding monenoic rather than the saturated fatty acids (61–63). There is no evidence that monenoic VLCFA are formed by the desaturation of the corresponding saturated VLCFA (54,64). Monenoic fatty acids depress the synthesis of saturated VLCFA (63,65–67), possibly by competing with the saturates for the elongases. Active synthesis of fatty acids with up to 34 carbon atoms occurs even in tissue which contains small amounts of endogenous monenoic VLCFA, indicating that the longer chain fatty acids are probably formed in most tissues (15).

Polyenoic VLCFA. Synthesis of polyenoic VLCFA has been described in a number of different mammalian tissues using either radiolabeled polyenoic fatty acids or acetate as precursors. Labeled polyenoic VLCFA are formed from [^{14}C]acetate and [^{14}C]arachidonic acid, respectively, by isolated rat spermatocytes and spermatids (33,34). [1-^{14}C]-Arachidonic acid injected into rat testis was also elongated *in vivo* to form n-6 tetra- and pentaenoic VLCFA with up to 30 carbon atoms (35). The longer chain fatty acids are formed by the sequential two carbon elongation of polyenoic fatty acid precursors, probably in much the same way as shorter chain fatty acids are synthesized. Rosenthal and Hill (55) reported the synthesis of n-6 polyenoic VLCFA from 20:3n-6 in endothelial cells in culture. These authors observed that most of the synthesized polyenoic VLCFA were in the culture medium in which the cells had been grown. Penta- and hexaenoic n-3 VLCFA synthesis from [1-^{14}C]acetate has also been reported in bovine retina (68).

Labeled 24 carbon fatty acids are formed from [^{14}C]-22:5n-3 and 22:4n-6 by skin fibroblasts and retinoblastoma cells (53,69). Tetraenoic fatty acids with up to 28 carbon atoms are synthesized from 20:4 and 24:4 by rat seminal tubules, but 32:4 is not elongated (39). Labeled tetra- and pentaenoic VLCFA with up to 30 carbon atoms are also detected when [U-^{14}C]acetate is used as the label. Surprisingly, labeled odd-chain tetraenoic VLCFA (21, 23, 25 and 27 carbon compounds) are also formed from [U-^{14}C]acetate (39). It has been speculated that the odd-chain compounds are formed by the α-oxidation of the corresponding precursor even-chain fatty acid. Synthesis of polyenoic VLCFA has also been observed in isolated human neutrophils. The carbon chains of [1-^{14}C]20:4n-6 and 24:4n-6 are extended by up to four carbon atoms (50) by these cells, but there is no extension in the carbon chain length of a longer chain tetraenoic fatty acid (30:4).

Apart from elongation of the carbon chain, tetraenoic VLCFA are also desaturated to form the corresponding pentaenoic fatty acids. Indeed, it is now believed that the desaturation of 24 carbon polyenoic fatty acids plays a pivotal role in the synthesis of 22 carbon n-3 hexaenoic fatty acids (52). Thus, the synthesis of 22:6n-3 takes place *via* a 4-desaturase-independent process as follows: 22:5n-3 → 24:5n-3 → 24:6n-3 → 22:6n-3, involving a desaturation of a polyenoic VLCFA (24:5n-3) by 6-desaturase followed by a subsequent oxidative step to generate the 22 carbon hexaenoic fatty acid. Studies of Aveldano *et al.* (39) provide evidence that 22 carbon n-6 fatty acids are formed by a similar pathway, i.e., 22:4n-6 → 24:4n-6 → 24:5n-6 → 22:5n-6 in rat seminal tubules, and may also apply in other tissues. These authors observed a very rapid synthesis of 24:5n-6 from [1-^{14}C]-24:4n-6. A number of odd- and even-chain tetra- and pentaenoic fatty acids with up to 30 carbon atoms were detected in the incubation mixtures, probably formed by elongation of 24:4 and 24:5 and chain shortening of the products.

Because of the presence of n-6 hexanoic, and even heptaenoic, VLCFA in some tissues, some desaturation of polyunsaturated fatty acids with >24 carbon atoms may also occur (20,46). The 11,14,17,20[1-^{14}C]26:4 injected intracerebrally into rats was elongated to form VLCFA with up to 36 carbon atoms (70). However, significant amounts of label were detected in 26:5n-6 and 28:5n-6, indicating that desaturation of the 26 carbon fatty acid can take place in brain. While it is possible that the labeling of pentaenoic VLCFA reflected the β-oxidation of 26:4 and subsequent incorporation of released

acetate into fatty acids, this seems unlikely in view of the relatively low β-oxidation activity observed in these tissues. It was not clear from this report where in the fatty acid molecule the desaturation had occurred. If the product formed was 8,11,14,17,20[1-^{14}C]26:5 [and this was not confirmed, but seems likely in view of the fact that the unlabeled fatty acid is a normal component of human and rat brain (20,48)], the enzyme acting on the fatty acid had 8-desaturase activity. However, as the identity of the product was not unambiguously determined, the possibility that the double bond was introduced at some other site in the carbon chain by another desaturase cannot be discounted. More recent metabolic studies in neutrophils and seminal tubules using even longer chain polyenoic VLCFA (i.e., 30:4n-6 and 32:4n-6) indicate that these fatty acids are poor substrates for desaturases (39,50). It is likely, therefore, that the final desaturation step in the synthesis of n-6 hexaenoic VLCFA in tissues such as brain involves either a 26 or 28 carbon pentaenoic acid. The product is then elongated to give VLCFA with 30–38 carbon atoms.

DEGRADATION

Saturated and monenoic fatty acids. Saturated fatty acids with up to 22 carbon atoms are degraded in mitochondria and peroxisomes. The movement of fatty acids across the double membranes of mitochondria and into the matrix where the oxidative process takes place is determined by a complex series of reactions involving acyl CoA synthetases (ligases), carnitine acyltransferase 1 (CPT1), translocase and CPT2 (71,72). The first enzyme in this sequence, acyl CoA synthetase, is specific for fatty acids with less than 24 carbon atoms (73,74) and is membrane-bound (75). Acyl CoA synthetase activity toward shorter chain saturated fatty acids, such as palmitic and lauric acids, is present in peroxisomal membranes (76), indicating that conversion of the fatty acid to its thioester may be necessary for peroxisomal import. Although Miyazawa *et al.* (77) reported that the acyl CoA synthetase activities in peroxisomes and mitochondria are due to the same enzyme protein, doubts remain as to whether the two organelles share the same enzyme.

On the other hand, there is now clear evidence that rat liver peroxisomes and microsomes contain an acyl CoA synthetase activity which acts on saturated VLCFA (73,74). Whereas the microsomal activity plays an integral role in lipid biosynthesis, the peroxisomal activity is an essential component of the peroxisomal β-oxidation pathway. It is not clear at present whether this enzyme (VLCFA CoA synthetase) is specific for saturates or whether it is capable of acting on monenoic and polyenoic VLCFA. The inability of highly purified rat liver mitochondria to oxidize VLCFA is believed to be due to the absence of this enzyme from the organelle (73,74). However, isolated rat liver peroxisomes, which contain VLCFA CoA synthetase activity, efficiently oxidize lignoceric acid. Other studies carried out with human liver and fibroblasts indicate that the oxidation of saturated VLCFA occurs in peroxisomes in human tissues as well (78,79). However, Veerkamp and Moerkerk (80) and Reubsaet *et al.* (81) have produced some evidence for the mitochondrial oxidation of lignoceric acid by rat and human tissues. These data were generated using homogenates and measuring lignoceric acid oxidation in the presence and absence of mitochondrial inhibitors. One possible explanation for the inhibitory effect of rotenone and antimycin on the oxidation of lignoceric acid by the tissue homogenates used by this group is that the fatty acid was converted to its CoA thioester by the microsomal VLCFA CoA synthetase present in their preparations. In contrast to the free acid, VLCFA CoA is oxidized by peroxisomes and mitochondria of rat liver and human skin fibroblasts (78,82).

While the data for the peroxisomal localization of VLCFA oxidation in rat liver and human skin fibroblasts is convincing, the subcellular site of VLCFA oxidation in brain remains unclear. H. Singh *et al.* (83) observed lignoceric acid oxidation activity in two distinct subcellular fractions isolated from rat brain. Analysis of various enzymological markers in these fractions indicated that both mitochondria and peroxisomes were able to oxidize lignoceric acid. Moreover, the specific activity of VLCFA CoA synthetase was similar in both subcellular compartments. More recent reports by I. Singh's group (84,85) and by Lageweg *et al.* (86) have not confirmed these findings. The former have observed that mitochondria isolated from rat brain have much lower lignoceric acid oxidation and VLCFA CoA synthetase activities, while Lageweg *et al.* (86) found that inhibition of mitochondrial palmitoyltransferase activity had little effect on lignoceric acid oxidation. It has been suggested that the lignoceric acid oxidation observed by H. Singh *et al.* (83) in mitochondrial fractions was due to the presence of myelin in these fractions (85), and that VLCFA was oxidized by mitochondria after conversion to its thioester by myelin VLCFA CoA synthetase. It is worth noting, however, that despite the apparently negligible contamination of I. Singh's mitochondrial fractions with myelin, substantial amounts of lignoceric acid oxidation activity still remained in their mitochondrial fractions. Furthermore, their data were generated using young rats instead of the adult rats used by H. Singh *et al.* (83). The possibility that there are differences in mitochondrial lignoceric acid oxidation with development cannot be discounted.

While it is now generally accepted that there is a peroxisomal synthetase specific for saturated VLCFA, there is little information as to whether the other enzymes which take part in the peroxisomal oxidation of VLCFA, i.e., acyl CoA oxidase, the bifunctional protein, and 3-ketothiolase, have a broad substrate specificity and are capable of acting on shorter chain fatty acids as well as VLCFA, or whether there are separate enzymes capable of acting only on VLCFA (87–90). There is evidence for the existence of three separate enzymes with acyl CoA oxidase activities toward long-chain fatty acids, 2-methyl chain fatty acids, and bile acids in rat liver (91–93). Interestingly, the two oxidases which act on fatty acids are able to act on lignoceric acid (93,94). In contrast, it is believed that there are only two acyl CoA oxidases in human liver—one enzyme is specific for long-chain fatty

acids and the other for 2-methyl fatty acids and bile acids (92). Data obtained from patients with isolated defects in acyl CoA oxidase indicate that the human enzyme can handle both long-chain and VLCFA. The available evidence suggests that there is a single peroxisomal bifunctional protein in rat liver (95). It is generally believed that this enzyme has a broad substrate specificity toward fatty acids and is capable of acting on VLCFA. There are two genes for 3-ketothiolases in rat liver coding for separate enzyme proteins (96), but, as yet, there is no indication that there is a separate 3-ketothiolase for VLCFA. Recently, a novel acyl CoA dehydrogenase with some activity toward longer chain fatty acids has been isolated from rat liver mitochondria (90). Although the enzyme has been referred to as a very long chain acyl CoA dehydrogenase to distinguish it from other mitochondrial acyl CoA synthetases, it is most active toward 16 and 18 carbon fatty acids. While the enzyme appears capable of acting on lignoceroyl CoA, its activity toward this substrate is much less (about 10%) than that observed with palmitoyl CoA (90). Moreover, because isolated rat liver mitochondria are unable to oxidize lignoceric acid (73), the metabolic role of this dehydrogenase is unclear.

Topographical studies on the localization of the active site of peroxisomal VLCFA synthetase have produced conflicting results. I. Singh's group (97) has proposed that the active site of the enzyme is on the lumenal surface of peroxisomes, while Lageweg *et al.* (98) have claimed that the enzyme is on the cytoplasmic side. The localization of VLCFA CoA synthetase is of considerable importance because of its implications in VLCFA oxidation. Because the peroxisomal oxidation of fatty acids is not enhanced by the addition of carnitine (99), it is believed that carnitine acyl CoA transferases are not necessary for the uptake of fatty acids into the organelle. Carnitine octanoyl CoA transferase has been reported to occur in peroxisomes, but it is thought to be involved in the elimination of octanoyl CoA, a product of fatty acid oxidation, from peroxisomes (100). However, the finding that the active site for VLCFA CoA synthetase is located on the lumenal side of the peroxisomal membrane (97) indicates that some mechanism for uptake of the free VLCFA must exist. This hypothesis is further supported by a report that uptake of VLCFA into peroxisomes from human skin fibroblasts is much greater than the uptake of the corresponding thioester (101). Moreover, the oxidation of unesterified lignoceric acid by isolated rat liver peroxisomes is also much greater than the oxidation of lignoceroyl CoA (101). It is tempting to speculate that VLCFA entry into peroxisomes is regulated by a peroxisomal membrane factor, possibly after the binding of the fatty acid to a binding protein. Fatty acid binding proteins (FABP) have been found in many different tissues (102,103). Peroxisomal proliferators induce the expression of FABP in rat liver and intestine (104) as well as in cultured rat hepatocytes, and also induce an increase in peroxisomal oxidation (105,106). Although a number of FABP have been described, there is, as yet, no evidence for the occurrence of a FABP which is specific for VLCFA.

Recent studies by Aubourg's group (107,108) have led to the identification of a protein which is involved in the oxidation of VLCFA. This protein belongs to the ATP-binding cassette (ABC) transmembrane transporter superfamily (109) and may be necessary for normal uptake of VLCFA into peroxisomes. However, it is doubtful that the protein is involved in this process as adenosine triphosphate (ATP) is normally required for normal function of other ABC transporter proteins, but is not required for VLCFA uptake into peroxisomes (101). It would seem more likely that the protein may play a role in the transport of enzyme proteins which take part in VLCFA oxidation.

There have been few reports on the degradation of monoenoic VLCFA. Human skin fibroblasts oxidize erucic (110, 111), tricosenoic and tetracosenoic (22:1, 23:1 and 24:1, respectively) acids (61,112), a large part of this process probably occurring in peroxisomes (110).

Polyenoic VLCFA. The number of studies on the oxidation of polyenoic VLCFA has been limited. Street *et al.* (54) observed that [1-^{14}C]24:4n-6 was oxidized by human skin fibroblasts. A number of radioactive products were detected including carbon dioxide, various water-soluble products and short-chain fatty acids. It was proposed that the main product of oxidation was acetate which was either oxidized by mitochondria or entered the Krebs cycle. Some of the acetate produced also appeared to take part in the *de novo* synthesis of fatty acids. Because of the reduced oxidation activity of peroxisome-deficient cells, it was speculated that the oxidation took place in peroxisomes. However, more recent studies in our laboratory have demonstrated that peroxisome-deficient fibroblasts in suspension can oxidize 24:4n-6, but not 24:0, indicating that the oxidation of 24:4 can take place in either peroxisomes or mitochondria (Table 1). The most likely explanation for the differences between 24:0 and 24:4n-6 oxidation is that 24:4n-6 can be converted to 24:4n-6 CoA at the mitochondrial outer membrane and can therefore enter mitochondria to take part in β-oxidation. Preliminary experiments in our laboratory have confirmed that rat liver mitochondrial

TABLE 1
$^{14}CO_2$ Production from [1-^{14}C] Fatty Acids by Cultured Skin Fibroblasts in Suspension[a]

	16:0	24:0	24:4n-6	24:4n-6
	(pmol $^{14}CO_2$/h/mg protein)			
Controls (n = 4)	521 ± 132 (353–678)	163 ± 51 (107–230)	283 ± 45 (245–336)	148 ± 31 (121–192)
Zellweger syndrome				
1	571	12	266	44
2	790	11	317	57
3	820	11	422	121

[a]$^{14}CO_2$ produced from [1-^{14}C] fatty acids by suspensions of skin fibroblasts was measured as described by Street *et al.* (64). The assays were performed in duplicate, and the data in control cells are presented as mean ± standard deviation. The data for Zellweger syndrome are an average of two observations. The range of values are shown in parentheses.

outer membranes are able to convert 24:4 to its corresponding thioester, although the identity of the ligase catalyzing this reaction has not been characterized (Singh, H., Beckman, K., and Poulos, A., unpublished observations). There are a number of possibilities: (i) the enzyme is specific for polyenoic VLCFA (and not for the shorter chain polyenoic fatty acids) and therefore essential for the degradation of this class of fatty acids; (ii) the enzyme may be specific for all VLCFA including saturated and polyenoic derivatives (although this seems unlikely in view of our findings that 24:4, but not 24:0, is a substrate for mitochondrial acyl CoA synthetase; and (iii) 24:4n-6 is a substrate for arachidonoyl CoA synthetase, an enzyme which has been reported to be quite distinct from the one acting on saturated long-chain fatty acids (113) and which acts on other tetraenoic fatty acids. Mitochondrial outer membranes are able to convert 24:4 CoA to the carnitine ester of 24:4 (Singh, H., and Poulos, A., unpublished data). These data are strongly suggestive of a mitochondrial role in the oxidation of 24:4, although it is still not known whether the longer chain polyenoic VLCFA (>24 carbons) can also be oxidized in this organelle. A polyenoic VLCFA such as 24:4 can be oxidized in both mitochondria and peroxisomes, but as the carbon chain length of a fatty acid increases, the role of peroxisomes probably becomes more important.

Some studies have been carried out with polyenoic VLCFA with carbon chain length greater than 24. Robinson *et al.* (70) injected [1-^{14}C]26:4 intracerebrally into rats and observed the formation of small amounts (0.5–1.6% of the total recovered label) of water-soluble products, indicating that some oxidation of the substrate had occurred. 24:4n-6 and 32:4n-6 have also been shown to be oxidized to carbon dioxide and water-soluble products by rat seminal tubules (39). However, oxidation of exogenous 32:4n-6 is much slower than 24:4n-6, possibly because of the limited uptake of the former into cells (39; Poulos, A., Beckman, K., and Singh, H., unpublished observations using cultured skin fibroblasts). Because of this difference in uptake, it cannot be concluded that 32:4n-6 is less metabolically active than 24:4n-6. As determined by measuring carbon dioxide and water-soluble metabolite formation, the oxidation of 24:4n-6 is greater than the oxidation of the shorter chain tetraenoic acid, arachidonic acid (39). One possible explanation is that a greater proportion of the arachidonic acid than of 24:4n-6 is incorporated into cellular phospholipids (and much of this into phosphatidylinositol) which, presumably, leaves less arachidonic acid available as a substrate for catabolic processes. In contrast, most of the exogenous 24:4n-6 was incorporated into triacylglycerol rather than into phospholipids. The triacylglycerol-linked fatty acid may be more likely to take part in degradative processes than the phospholipid-bound fatty acid. The lower incorporation of the longer chain polyenoic VLCFA has also been observed in human neutrophils (50).

Polyenoic fatty acids with long and very long chains having an odd number of carbon atoms occur as endogenous components of reproductive tissue lipids (36,37,39). Labeling experiments using [U-^{14}C]acetate have demonstrated that rat seminal tubules are able to generate these peculiar VLCFA *in situ* (39). The metabolic origin of the odd-chain polyenoic VLCFA remains to be determined. It has been proposed that they may arise by the one carbon shortening of even carbon chain fatty acids, presumably by an α-oxidative process. Despite intensive study over the last thirty years, the mechanism of α-oxidation has not yet been elucidated (114–119). The possibility that the oxidation of some of the polyenoic VLCFA of a given tissue proceeds *via* α-oxidation is intriguing and worthy of further investigation.

Additional enzymes, such as Δ^3, Δ^2-enoyl-CoA isomerase and 2,4-dienoyl CoA reductase, are required for the breakdown of polyenoic fatty acids (120,121). The peroxisomal bifunctional protein has been shown to have trifunctional activity possessing Δ^3, Δ^2-enoyl isomerase, as well as 2-enoyl CoA hydratase and 3-hydroxyacyl CoA dehydratase activities (121). It is likely that the isomerase and reductase are also necessary for the oxidation of the corresponding polyenoic VLCFA.

THE INVOLVEMENT OF VERY LONG CHAIN FATTY ACIDS IN HUMAN DISEASE

Saturated VLCFA. Igarashi *et al.* (122,123) were the first to report on the apparent link between VLCFA and human disease. They observed the accumulation of saturated VLCFA in post-mortem tissues of patients with X-linked adrenoleukodystrophy (ALD), a rare neurodegenerative disorder affecting young males. Brain cholesterol esters and gangliosides, and adrenal cholesterol esters, were found to have large amounts of odd- and even-carbon chain length saturated VLCFA, in particular 25:0 and 26:0 fatty acids. These findings were later confirmed by other groups (124–126). These VLCFA are also present in many other ALD tissues or body fluids including plasma (14), muscle (127), cultured skin fibroblasts (128), cultured aminocytes (129), erythrocytes (23) and leukocytes (130). More recent investigations have revealed that the fatty acids which accumulate in brain are mixtures of saturated and monenoic derivatives with carbon chain lengths up to 34 (20,131), the monenoic fatty acids comprising both n-7 and n-9 derivatives. While the saturated VLCFA occur largely in cholesterol esters, the monenoic VLCFA are found mainly in molecular species of phosphatidylcholine which are partially separable by thin-layer chromatography from other molecular species of the phospholipid occurring in brain (20).

Because of the belief that the inflammatory response which leads to the extensive demyelination observed in ALD brain (132,237) is in some way related to the accumulation of saturated VLCFA, attempts have been made to correlate the pathological changes with VLCFA levels. Reinecke *et al.* (133) reported that the severity of the pathological changes in the different parts of the brain appeared to correlate with the saturated VLCFA levels in the cholesterol ester fraction, and speculated that the VLCFA changes preceded the inflammatory response. A similar study was conducted by Theda *et al.*

(131) who reported that there was an increase in the levels of VLCFA-containing glycerolipids, and particularly phosphatidylcholine (VLCFA–PC), in the actively demyelinating edge of the lesions and, apparently, in regions of the brain. They suggested that these molecular species of VLCFA–PC may be antigenic and could therefore provide a focus for an immunological attack of the brain (133).

The accumulation of VLCFA is paralleled by the reduced ability of ALD patients to oxidize VLCFA to acetate and carbon dioxide (82,134–137). The decrease in fatty acid oxidation appears to be related to a reduced activity of peroxisomal VLCFA CoA synthetase activity (138,139). More recently, the primary defect in ALD has been found to be in a gene coding for peroxisomal protein which belongs to the ABC superfamily of transporters (107,108). The relationship between the defect in a transporter protein and an abnormality in VLCFA oxidation has yet to be determined. As mentioned earlier, the protein may be involved in the transport of VLCFA into peroxisomes. Alternatively, it may be required either for the appropriate insertion of VLCFA CoA synthetase into peroxisome membranes, or to stabilize the synthetase once it has been inserted into the peroxisomal membrane. Using immunofluorescent techniques, it has been shown that the ALD protein is located in peroxisomes (Aubourg, P., private communication).

In addition to the impairment in VLCFA oxidation, ALD skin fibroblasts show an increase in fatty acid elongation activity (64,140–143). However, the biochemical basis for this remains unknown. It has been suggested that the increased elongation simply reflects the greater availability of VLCFA due to the impaired oxidation activity (64). These findings are consistent with a possible role of peroxisomes in the regulation of microsomal fatty acid elongation, but precisely how this regulation occurs is not known.

The presumed involvement of saturated VLCFA in the pathogenesis of ALD raised the possibility of treatment of the disease by restriction of the dietary intake of these fatty acids, particularly as it was shown that a significant proportion of the VLCFA which accumulates in ALD brain is of dietary origin (144). The use of a VLCFA-restricted diet (145) was unsuccessful in reducing the plasma levels of these fatty acids, and did not offer any clinical benefits (146). This suggested that considerable synthesis of saturated VLCFA, presumably by elongation of shorter chain fatty acid precursors, takes place in patient tissues. Oleic acid was later shown to inhibit the synthesis of saturated VLCFA in skin fibroblasts in culture, possibly by competing with the endogenous shorter chain saturated fatty acids for the microsomal elongases involved in fatty acid synthesis (66). However, oleic acid (as glycerol trioleate) was not totally effective in reducing plasma levels of saturated VLCFA (147,148). A much greater reduction in saturated VLCFA levels was achieved by supplementing the diet with glycerol trioleate and glycerol trierucate (glycerol esterified to erucic acid, 22:1n-9), the so-called "Lorenzo's Oil" (65,67,149), but the clinical benefits, at least for patients with neurological signs, do not appear to be very significant (150,151). Plasma and liver of ALD patients who have received dietary supplementation with "Lorenzo's Oil" show increased levels of erucic acid and its elongation products (24:1 and 26:1). However, while an early study indicated that erucic acid was able to cross into the brain (67), these findings have not been confirmed (152–154) and raise serious doubts as to the capacity of the diet to alter the VLCFA composition of the brain and thereby produce clinical benefits. Whether greater amounts of erucic acid would be able to cross into the brain in younger, neurologically-intact patients is under current investigation.

In the last few years a number of other peroxisomal disorders which are also characterized by accumulation of saturated VLCFA in plasma and cultured skin fibroblasts have also been described. These patients may be divided into those who have normal numbers of morphologically-normal peroxisomes, but who have an abnormality in any one of a number of peroxisomal enzymes involved in β-oxidation (group 1), and those whose tissues show a greatly reduced number and/or morphologically-abnormal peroxisomes (group 2). The former group includes X-linked ALD and also includes patients with acyl CoA oxidase, bifunctional protein and 3-ketothiolase deficiency states (155–159). The latter group includes patients with Zellweger's syndrome, the neonatal autosomal recessive form of ALD, infantile Refsum's disease and various other conditions (160–166).

Unsaturated VLCFA. Saturated VLCFA are increased in the plasma of group 1 and 2 patients, but the latter in particular also show elevations in monounsaturated VLCFA (136,167). These are mainly 25, 26 and 28 carbon fatty acids and are a mixture of the n-7 and n-9 series. Some patients with group 2 disorders, notably those with Zellweger's syndrome, also accumulate polyenoic VLCFA in plasma (51). Elevated amounts of 24:5n-6, 26:5n-6, 28:5n-6, 30:5n-6 and 30:6n-6 have been detected in the plasma of Zellweger's syndrome patients, and in the brain of this and other diseases of group 2 (20,46). These studies indicated that polyenoic VLCFA accumulate only in the plasma of patients with defects in peroxisomal assembly, and specifically in those individuals who have the clinical features of Zellweger's syndrome. However, somatic cell hybridization studies have demonstrated that there are at least eight different genes involved in peroxisomal assembly (168). Mutations in any one of these genes can affect peroxisomal assembly and cause disease. Zellweger's syndrome may result from an abnormality in any one of at least five of these genes. Recently, it has been shown that one of these genes codes for a 35 kDa peroxisomal integral membrane protein (238). The involvement of a gene coding for a 70 kDa protein, a member of the ATP-binding transporter family, in the most common complementation group, is not so clear-cut (239). Zellweger's syndrome patients may belong to the same complementation groups (i.e., have different abnormalities in the same gene) as other peroxisome-deficient patients (e.g., neonatal ALD and infantile Refsum's disease) whose clinical presentations are quite different. Plasma polyenoic VLCFA are not normally increased

in neonatal ALD and infantile Refsum's disease patients, although there is an increase in the levels of polyenoic VLCFA in the brain of these patients (20). It is not known why these fatty acids do not accumulate in non-Zellweger's syndrome plasma. One likely explanation is that the plasma polyenoic VLCFA levels are only increased in the most severely affected patients, a hypothesis which has received some support from ultrastructural studies showing the virtually complete absence of peroxisomes in the liver of many Zellweger's syndrome patients and some peroxisomal structures in non-Zellweger's syndrome patients (166). The difference may also be related to the fact that Zellweger's syndrome patients are generally diagnosed in the first few days or weeks of life, while, for other phenotypes, diagnosis may not be made until later in infancy (169–171).

Studies carried out on the brains of patients with peroxisomal assembly defects have demonstrated that the most pronounced change occurs in the hexaenoic VLCFA. These fatty acids are not detected in normal infant or adult brain, but occur in trace amounts in fetal brain (20). Hexaenoic VLCFA are also found in small amounts in the brains of very young rats (one-day-old), but, as in human brain, they are undetectable in older animals (48). The accumulation of the very long chain polyenes in peroxisomal disease may be, at least in part, a consequence of the reduced capacity of the brain to oxidize them.

The increased activity of microsomal fatty acid carbon chain elongation may also contribute to the accumulation of unsaturated VLCFA in group 2 patients. Radiolabeled monenoic VLCFA with up to 34 carbon atoms are formed from [2-^{14}C]acetate in greater amounts than those observed in control cell lines (15). They probably arise by elongation of shorter chain (monoenoic) fatty acids. While the synthesis of the monenoic VLCFA may have been increased because of the decreased β-oxidation activity, microsomal elongation activity is also increased in these cells (141).

The observation that the levels of polyenoic VLCFA are not greatly altered in X-linked ALD brain is surprising, particularly in view of the reported increased elongation activity in ALD tissue (141,143). As monenoic VLCFA are increased in ALD, it is probable that the increased elongation activity is specific for saturates and monounsaturates and does not act on polyunsaturates. This is consistent with the normal elongation of 24:4n-6 observed in ALD fibroblasts (64). One interpretation of these data is that a specific elongase (or elongases) take part in the biosynthesis of polyenoic VLCFA. The normal elongation of 24:4n-6 by ALD fibroblasts would tend to support this view.

FUNCTION

Little is really known about the possible function of VLCFA in animal tissues. However, while recent studies have not provided all, or even most, of the answers, they have been particularly useful in better defining the metabolic role of these lipids. These studies have, in turn, provided a firmer theoretical basis for speculation about function.

Structural role. A structural role for VLCFA was inferred many years ago with the recognition of the presence in very high abundance of saturated and monenoic VLCFA in the myelin sheath (6,172,173). It has been suggested that the VLCFA increase the stability of myelin (56). Similarly, a structural role has been suggested for the n-3 and n-6 polyenoic VLCFA (which are esterified to the *sn*-1 position of phosphatidylcholine) in the photoreceptor membranes of vertebrate retina (44). The importance of fatty acids such as docosahexaenoic acid in the maintenance of normal visual function has been established (174), but the role of the polyenoic VLCFA has not yet been studied. It has been proposed that the thermal behavior of the retinal rod inner outer segments reflects the asymmetric distribution of lipids in these membranes (175). Presumably the interaction of lipids with the various protein elements in membranes contributes to the properties of the membrane structures. Certainly, there is good evidence for the ability of lipids to influence the conformation of peptides (176). Because the molecular species of polyenoic VLCFA-containing phosphatidylcholine remains associated with rhodopsin even after repeated extraction with hexane, it has bene postulated that they may partially surround the α-helix of rhodopsin, thereby providing an environment necessary for normal function of the photoreceptor membranes (44). VLCFA are also highly concentrated in spermatozoa, only in that case they are bound to sphingomyelin. Experiments in our laboratory indicate that such sphingomyelins are distributed in the head, tail and midpiece, as well as in the lipoprotein coat in ram spermatozoa. As the sequential actions of sphingomyelinase and ceramidase are probably involved in the release of the VLCFA from sphingomyelin (177), and as the activity of ceramidase in comparison to other lysosomal enzymes is generally low in mammalian tissues (178,179), the turnover of sphingomyelin is probably slower than that of glycerophospholipids, which supports the idea of a structural role for VLCFA-containing sphingomyelins. However, recent reports of the distribution of polyenoic VLCFA in glycerolipids, and the identification of hydroxylated polyenoic VLCFA in rat spermatozoa (39), suggest that VLCFA may have multiple functions in spermatozoa.

Another possible structural role for VLCFA has been suggested from their presence in the lipids of meibomian glands, skin and hair of mammals. The meibomian glands are located within the eyelids of mammals, producing an oily secretion which forms a layer on the surface of the aqueous tear film and believed to contribute to its stability (180–182). The lipids in the secretions from the meibomian glands are thought to act as a barrier to the entry of skin surface lipids which could disrupt the tear film, prevent the spillover of the tear film, reduce evaporation, and may have some limited function as a lubricant (180). The VLCFA in the skin may also act as a barrier, although in this case it seems likely that they are involved in the prevention of transepidermal water loss (183). The unusual epidermal lipids, O-acylceramide and O-acylglucosyl ceramide, which contain linoleic acid moi-

eties esterified to the ω-hydroxyl groups of ω-hydroxyl monoenoic VLCFA, are thought to be necessary to ensure the maintenance of an adequate hydrophobic barrier to the movement of water (19,25). The importance of these lipids is suggested by the transepidermal loss of water in individuals who are essential fatty acid-deficient (184–185).

Metabolic role. A possible metabolic role, particularly for the 24 carbon n-6 tetraenoic and 24 carbon n-3 pentaenoic fatty acids, in biosynthesis of the important 22 carbon polyenoic fatty acids (52) (22:5n-6 and 22:6n-3) has been discussed earlier. It has also been demonstrated that the terminal two carbons of the saturated, and particularly the polyenoic VLCFA, once cleaved can be used for the cytoplasmic *de novo* synthesis of shorter chain fatty acids (54,64), although it is possible that some synthesis of fatty acids may occur in peroxisomes (186). As labeled amino acids may be formed from carboxy-labeled VLCFA (54,64), it is probable that acetate formed by β-oxidation enters the tricarboxylic acid (TCA) cycle where it is utilized for various biosynthetic processes. Acetate is also converted to acetoacetate in peroxisomes and utilized for the synthesis of sterols (187,188). In this context it is worth noting that both sterol carrier protein-2, which participates in cholesterol and bile acid biosynthesis (189,190), and 3-hydroxy-3-methylglutaryl CoA reductase (191), the key regulatory enzyme in cholesterol biosynthesis, are also present in peroxisomes. Presumably some of the acetate derived from the peroxisomal β-oxidation of fatty acids is transported in some form either to the cytosol where it takes part in fatty acid synthesis, or to the endoplasmic reticulum where it is used for carbon chain elongation of fatty acids, or into the mitochondria where it joins the TCA cycle. How this is done, and what determines where the released acetate is sent in the cell remains a mystery. The further issue of whether the acetate formed from VLCFA has some other, as yet unknown function, is not known. Certainly, a defect along the peroxisomal β-oxidation pathway affecting the metabolism of VLCFA appears to have devastating clinical effects on patients seemingly out of all proportion to the plasma levels of VLCFA (155,157). While it is plausible that the clinical effects relate to the toxicity of the VLCFA (192,149), the possibility that VLCFA β-oxidation has some other critical cellular function cannot be discounted. Cell lines which are deficient in peroxisomal β-oxidation also have disturbances in other parts of the cell. For example, the activities of microsomal fatty acid elongases are increased (141), and there may also be disturbances in desaturase activity (193,194). Moreover, there are morphological changes in mitochondria from some patients (160,195,196).

VLCFA can be precursors of other molecules such as alkanes (197), alcohols, or aldehydes. As yet, however, there is no indication that such products contribute to the pathology of peroxisomal disease. Because of the possibility that polyenoic VLCFA are converted into oxygenated metabolites in a manner similar to that observed for the shorter chain polyenoic fatty acids (198–200), and these oxygenated VLCFA may induce some of the pathological changes observed in peroxisomal disease, we have analyzed the products formed by incubating polyenoic VLCFA with different cells. Human neutrophils are able to convert 24:4n-6 into its 9-hydroxylated derivative in a reaction presumably catalyzed by 5-lipoxygenase (50,201), but cyclooxygenases do not act on this fatty acid. However, the longer carbon chain length VLCFA (e.g., 30:4n-6) are not substrates for neutrophil lipoxygenases. Our data indicate that 30 and 32 carbon tetraenoic fatty acids are poorly taken up into cells. They are mainly incorporated into diacylglycerol and triacylglycerols in fibroblasts (Poulos, A., Beckman, K., and Singh, H., unpublished data), neutrophils (50) and seminal tubules (39).

Signal transduction. The role of lipids in cellular signalling has received increasing attention since the important discoveries of Nishizuka (202) and Majerus *et al.* (203) in the mid-1980s. These workers reported the release of diacylglycerol and inositol phosphates in response to the binding of an agonist to its membrane receptor and the subsequent activation of phospholipase C. Diacylglycerol and inositol phosphates act as second messengers, activating protein kinase C and releasing intracellular stores of calcium, respectively. In the last few years it has become apparent that agonist-induced hydrolysis of phosphatidylcholine (204–206) and phosphatidylethanolamine (207–209) also occur. It has also become clear that agonists may induce the release of phosphatidic acid *via* the activation of phospholipase D. Phosphatidic acid is believed to produce its effect after conversion to diacylglycerol by phosphatidic acid phosphohydrolase (210–212) or lysophosphatidic acid (213), or it may be active in its own right (214). In addition to the involvement of the glycerophospholipids, it has become apparent that sphingolipids also play a role in cellular signalling (215). The binding of an agonist such as tumor necrosis factor α (TNFα) to its membrane induces the hydrolysis of sphingomyelin and the concomitant release of ceramide, an important second messenger of the sphingolipid cycle (216–218). Ceramide induces its effect *via* its action on ceramide-activated protein phosphatase (219,220) or protein kinase (221). Other sphingolipids, including ceramide-1-phosphate, sphingosine-1-phosphate and sphingosine itself, also take part in cellular signalling processes (215). As phospholipases A_2, C and D, and sphingomyelinase have all been reported to be activated in response to the binding of various agonists to membrane receptors (202,210,215,222), it is likely that VLCFA-containing second messenger lipids may be formed, particularly in cells (such as those of brain, retina and testis) which are rich in VLCFA. As yet it is not known whether any of the above cellular signalling processes can be influenced by the introduction of VLCFA ester or amide functions into the various lipid second messenger molecules. However, fatty acid structure can also influence the diacylglycerol and phosphatidylcholine-induced *in vitro* activation of protein kinase C (223), and lipid structure is also an important factor in the ability of protein kinase C to bind to lipid vesicles (224). If unique molecular species of lipid second messengers are indeed formed, it remains to be determined whether they play a role in signal transduction.

Unesterified fatty acids have also been shown to have potent physiological effects on cells. For example, the n-6 and n-3 polyunsaturated fatty acids such as arachidonic acid and docosahexaenoic acid activate certain cellular functions such as the oxygen-dependent respiratory burst in neutrophils (225–227) and macrophages (228), leukocyte chemotaxis (229), and neutrophil adhesion (230) by mechanisms which are not believed to involve eicosanoids. These effects indicate that fatty acids are able to modulate cellular signalling processes, and, indeed, they have been considered to be potential second messengers (231), but their mechanisms of action are not understood. The n-3 and n-6 polyenoic VLCFA, but not the corresponding saturates and monounsaturates, are able to activate the neutrophil respiratory burst (226,232), their activity declining with increasing carbon chain length. Although they appear to be less active than the shorter chain analogues, this may simply reflect their slower cellular uptake (39,50,232). It is unlikely that these effects are mediated by hydroxylated products because the longer carbon chain fatty acids (30 and 32 carbon fatty acids) are active although they are not substrates for neutrophil lipoxygenases and cyclooxygenases (50). We have recently observed that 30 carbon polyenoic fatty acids are able to induce calcium mobilization in isolated human neutrophils (Hardy, S.J., Robinson, B.S., Ferrante, A., Johnson, D.W., Poulos, A., and Murray, A.W., unpublished observations). As the 30 to 34 carbon polyenoic fatty acids are also very effective *in vitro* activators of protein kinase C, it is tempting to speculate that they may be involved in modulating the activity of this important regulatory enzyme (233).

CONCLUSIONS

The recognition of the involvement of VLCFA in human disease has prompted a renewed interest in this class of fatty acids. The inference to be drawn from the clinical observations is that VCLFA are important cellular components and that disturbances in their metabolism can have devastating consequences. Future studies of these intriguing lipids are likely to be fruitful in a number of different areas. Two of the most interesting questions concern the involvement of VLCFA in the neuropathology of peroxisomal disease and, in particular, in the severe demyelination which is a characteristic feature of X-linked ALD, and the role of the ALD protein in VLCFA oxidation. Other questions which will be addressed relate to the existence of enzymes and binding proteins which are specific for VLCFA. Thus far, there is evidence for the existence of at least one enzyme which is specific for VLCFA, e.g., VLCFA CoA synthetase, but it is probably that other enzymes will be identified in the future. The relationship between the mitochondrial and peroxisomal roles in the oxidation of VLCFA, and the precise mechanisms by which the products of these oxidative processes are channeled to the endoplasmic reticulum, presumably for use in biosynthetic processes, will also be explored. Finally, the exciting discoveries in signal transduction in the last few years have raised the possibility that the VLCFA, or VLCFA-containing lipids, have a role as messenger lipids. The development of greatly improved methods for the solubilization of VLCFA (234), and the availability of unlabeled and radiolabeled VLCFA (235,236), will provide a stimulus for these future investigations.

ACKNOWLEDGMENTS

This work was supported in part by the National Health and Medical Research Council of Australia, the Women's and Children's Hospital Foundation, and the Channel 7 Children's Research Foundation of South Australia. The author would like to thank Peta Knapman and Sophie Lazenkas for the word processing.

REFERENCES

1. Rezanka, T. (1989) *Progr. Lipid Res. 28*, 147–187.
2. Poulos, A. (1989) *Prog. Lipid Res. 28*, 35–51.
3. Pullarkat, R.K., and Rhea, H. (1976) *J. Neurochem. 27*, 321–322.
4. Johnson, D., Beckman, K., Fellenberg, A.J., Robinson, B.S., and Poulos, A. (1992) *Lipids 27*, 177–180.
5. Stallberg-Stenhagen, S., and Svennerholm, L. (1965) *J. Lipid Res. 6*, 146–155.
6. Svennerholm, L., and Stallberg-Stenhagen, S. (1968) *J. Lipid Res. 9*, 215–225.
7. Giusto, N.M., Roque, M.E., and Ilincheta de Boschero, M.G. (1992) *Lipids 27*, 835–839.
8. Nicholaides, N., Kaitaranta, J.K., Radah, T.N., Macy, J.I., Boswell, F.M., and Smith, R.E. (1981) *Invest. Opthalmol. Visual Sci. 20*, 522–536.
9. Tiffany, J.M. (1978) *Exp. Eye Res. 27*, 289–300.
10. Tiffany, J.M. (1979) *Exp. Eye Res. 29*, 195–202.
11. Harvey, D.J., and Tiffany, J.M. (1984) *J. Chromatogr. 301*, 173–187.
12. Harvey, D.J., Tiffany, J.M., Deurden, J.M., Pandher, K.S., and Mengher, L.S. (1987) *J. Chromatogr. 414*, 253–263.
13. Nicholaides, N., Santos, E.C., and Papadakis, K. (1984) *Lipids 19*, 264–267.
14. Moser, H.W., Moser, A.B., Frayer, K.K., Winston, W.W., Schulman, J.D., O'Neill, B.P., and Kishimoto, Y. (1981) *Neurology 31*, 1241–1249.
15. Poulos, A., Usher, S., and Paton, B. (1993) *Lipids 28*, 97–102.
16. Karlsson, K.A., Nilsson, K., Samuelsson, B.E., and Steen, G.O. (1969) *Biochim. Biophys. Acta 176*, 660–663.
17. Karlsson, K.A., Samuelsson, B.E., and Steen, G.O. (1973) *Biochim. Biophys. Acta 316*, 317–335.
18. Wertz, P.W., and Downing, D.T. (1983) *J. Lipid Res. 24*, 753–758.
19. Bowser, P.A., Nugteren, D.H., White, R.J., Houtsmuller, U.M.T., and Prottey, C. (1985) *Biochim. Biophys. Acta 834*, 419–428.
20. Sharp, P., Johnson, D., and Poulos, A. (1991) *J. Neurochem. 56*, 30–37.
21. Tsuji, S., Sano, T., Ariga, T., Sekine, M., Kuriyama, M., and Miyatake, T. (1981) *J. Neurochem 36*, 1046–1049.
22. Ramsey, R.B., Banik, N.L., and Davison, A.N. (1977) *J. Neurol. Sci. 29*, 277–294.
23. Antoku, Y., Sakai, T., Goto, I., Iwashita, H., and Kuroiwa, Y. (1984) *Neurology 34*, 1499–1501.
24. de Vries, G.H., and Norton, W.T. (1974) *J. Neurochem. 22*, 251–257.
25. Nugteren, D.H., Christ-Hazelhof, E., van der Beek, A., and Houtsmuller, U.M.T. (1985) *Biochim. Biophys. Acta 834*, 429–436.

26. Bizzozero, O.A., and Good, L.K. (1990) *J. Neurochem. 55*, 1986–1992.
27. Bizzozero, O.A., Zuniga, G., and Lees, M.B. (1991) *J. Neurochem. 56*, 872–878.
28. Bizzozero, O.A., Good, L.K., and Evans, J.E. (1990) *Biochem. Biophys. Res. Comm. 170*, 375–382.
29. Wertz, P.W., and Downing, D.T. (1987) *Biochim. Biophys. Acta 917*, 108–111.
30. Wertz, P.W., Madison, K.C., and Downing, D.T. (1989) *J. Invest. Dermatol. 92*, 109–111.
31. Davis, J.T., Bridges, R.B., and Coniglio, J.G. (1966) *Biochem. J. 98*, 342–346.
32. Bridges, R.B., and Coniglio, J.G. (1970) *J. Biol. Chem. 245*, 46–49.
33. Grogan, W.M., and Lam, J.W. (1982) *Lipids 17*, 604–611.
34. Grogan, W.M., and Heath, E.G. (1983) *Lipids 18*, 275–284.
35. Grogan, W.M. (1984) *Lipids 19*, 341–346.
36. Poulos, A., Sharp, P., Johnson, D.W., White, I.G., and Fellenberg, A.J. (1986) *Biochem. J. 240*, 891–895.
37. Poulos, A., Johnson, D.W., Beckman, K., White, I.G., and Easton, C. (1987) *Biochem. J. 248*, 961–964.
38. Aveldano, M.I., Rotstein, N.P., and Vermouth, N.T. (1992) *Biochem. J. 283*, 235–251.
39. Aveldano, M.I., Robinson, B.S., Johnson, D.W., and Poulos, A. (1993) *J. Biol. Chem. 268*, 11663–11668.
40. Robinson, B.S., Johnson, D.W., and Poulos, A. (1992) *J. Biol. Chem. 267*, 1746–1751.
41. Aveldano, M.I., Rotstein, N.P., and Vermouth, N.T. (1992) *Lipids 27*, 676–680.
42. Aveldano, M.I. (1987) *J. Biol. Chem. 262*, 1172–1179.
43. Aveldano, M.I., and Sprecher, H. (1987) *J. Biol. Chem. 262*, 1180–1186.
44. Aveldano, M.I. (1988) *Biochemistry 27*, 1129–1139.
45. Poulos, A., Sharp, P., Johnson, D., and Easton, C. (1988) *Biochem. J. 253*, 645–650.
46. Poulos, A., Sharp, P., Singh, H., Johnson, D., Fellenberg, A.J., and Pollard, A.C. (1986) *Biochem. J. 235*, 607–610.
47. Sharp, P., Poulos, A., Fellenberg, A.J., and Johnson, D. (1987) *Biochem. J. 248*, 61–67.
48. Robinson, B.S., Johnson, D.W., and Poulos, A. (1990) *Biochem. J. 265*, 763–767.
49. Poulos, A., Beckman, K., Johnson, D., Paton, B., Robinson, B.S., Sharp, P., Usher, S., and Singh, H. (1992) *Adv. Exp. Med. Biol. 318*, 331–340.
50. Robinson, B.S., Johnson, D.W., Ferrante, A., and Poulos, A. (1994) *Biochim. Biophys. Acta 1213*, 325–334.
51. Poulos, A., Sharp., P., and Johnson, D. (1989) *Neurology 39*, 44–47.
52. Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H. (1991) *J. Biol. Chem. 266*, 19995–20000.
53. Rosenthal, M.D., Garcia, M.C., Jones, M.R., and Sprecher, H. (1991) *Biochim. Biophys. Acta 1083*, 29–36.
54. Street, J.M., Johnson, D.W., Singh, H., and Poulos, A. (1989) *Biochem. J. 260*, 647–655.
55. Rosenthal, M.D., and Hill, J.R. (1984) *Biochim. Biophys. Acta 795*, 171–178.
56. Bourre, J.M. (1980) in *Neurological Mutations Affecting Myelination*, (Baumann, N., ed.) INSERM Symposium No. 14, pp. 187–205, Elsevier/North Holland Biomedical Press, Amsterdam.
57. Anderson, G.J., and Kolattukuddy, P.E. (1985) *Arch. Biochem. Biophys. 237*, 177–185.
58. Goldberg, I., Shechter, I., and Bloch, K. (1973) *Science 182*, 497–499.
59. Yoshida, S., and Takeshita, M. (1987) *Arch. Biochem. Biophys. 254*, 170–179.
60. Kugi, M., Yoshida, S., and Takeshita, M. (1990) *Biochim. Biophys. Acta 1043*, 83–90.
61. Bourre, J.M., Daudu, O., and Baumann, N. (1976) *Biochim. Biophys. Acta 424*, 1–7.
62. Saitoh, T., Yoshida, S., and Takeshita, M. (1988) *Biochim. Biophys. Acta 960*, 410–416.
63. Wilson, R., Tocher, D.R., and Sargent, J.R. (1992) *J. Neurol. Sci. 109*, 207–214.
64. Street, J.M., Singh, H., and Poulos, A. (1990) *Biochem. J. 269*, 671–677.
65. Odone, A., and Odone, M. (1989) *J. Pediatr. Neurosci. 5*, 55–61.
66. Rizzo, W.B., Watkins, P.A., Phillips, M.W., Cranin, D., Campbell, B., and Avigan, J. (1986) *Neurology 36*, 357–361.
67. Rizzo, W.B., Leshner, R.T., Odone, A., Dammann, A.L., Craft, D.A., Jensone, M.E., Jennings, S.S., Davis, S., Jaitly, R., and Sgro, J.A. (1989) *Neurology 39*, 1415–1422.
68. Rotstein, N.P., and Aveldano, M.I. (1988) *Biochem. J. 249*, 191–200.
69. Garcia, M.C., Sprecher, H., and Rosenthal, M.D. (1990) *Lipids 25*, 211–215.
70. Robinson, B.S., Johnson, D.W., and Poulos, A. (1990) *Biochem J. 267*, 561–564.
71. Bremer, J. (1983) *Physiol. Rev. 63*, 1420–1480.
72. Bieber, L.L. (1988) *Ann. Rev. Biochem. 57*, 261–283.
73. Singh, H., Derwas, N., and Poulos, A. (1987) *Arch. Biochem. Biophys. 259*, 382–390.
74. Singh, H., and Poulos, A. (1988) *Arch. Biochem. Biophys. 266*, 486–495.
75. Krisans, S.K., Mortenson, R.M., and Lazarow, P.B. (1980) *J. Biol. Chem. 255*, 9599–9607.
76. Shindo, Y., and Hashimoto, T. (1978) *J. Biochem. (Tokyo) 84*, 1177–1181.
77. Miyazawa, S., Hashimoto, T., and Yokota, S. (1985) *J. Biochem. (Tokyo) 98*, 723–733.
78. Singh, H., Derwas, N., and Poulos, A. (1987) *Arch. Biochem. Biophys. 257*, 302–314.
79. Jakobs, B.S., and Wanders, R.J.A. (1991) *Biochem. Biophys. Res. Commun. 178*, 842–847.
80. Veerkamp, J.H., and Moerkerk, H.T.B. (1986) *Biochim. Biophys. Acta 875*, 301–310.
81. Reubsaet, F.A.G., Veerkamp, J.H., Trijbels, J.M.F., and Monnens, L.A.H. (1989) *Lipids 24*, 945–950.
82. Singh, H., Derwas, N., and Poulos, A. (1987) *Arch. Biochem. Biophys. 254*, 526–533.
83. Singh, H., Usher, S., and Poulos, A. (1989) *J. Neurochem. 53*, 1711–1718.
84. Lazo, O., Singh, A.K., and Singh, I. (1991) *J. Neurochem. 56*, 1343–1353.
85. Singh, I., Lazo, O., and Kremser, K. (1993) *Biochim. Biophys. Acta 1170*, 44–52.
86. Lageweg, W., Sykes, J.E.C., Lopes-Cardozo, M., and Wanders, R.J.A. (1991) *Biochim. Biophys. Acta 1085*, 381–384.
87. Miyazawa, S., Hayashi, H., Hijakata, M., Ishii, N., Furuta, S., Kagamiyama, H., Osumi, T., and Hashimoto, T. (1987) *J. Biol. Chem. 262*, 8131–8137.
88. Ishii, N., Hijikata, M., Osumi, T., and Hashimoto, T. (1987) *J. Biol. Chem. 262*, 8144–8150.
89. Hijakata, M., Ishii, N., Kagamiyama, O.H., Osumi, T., and Hashimoto, T. (1987) *J. Biol. Chem. 262*, 8151–8158.
90. Hashimoto, T. (1992) *Progr. Clin. Biol. Res. 375*, 19–32.
91. Schepers, L., Van Veldhoven, P.P., Casteels, M., Eyssen, H.J., and Mannaerts, G.P. (1990) *J. Biol. Chem. 265*, 5242–5246.
92. Van Veldhoven, P.P. Vanhove, G., Vanhoutte, F., Dacremont, G., Parmentier, G., Eyssen, H.J., and Mannaerts, G.P. (1991) *J. Biol. Chem. 266*, 24676–24683.
93. Van Veldhoven, P.P., Vanhove, G., Asselberghs, S., Eyssen,

H.J., and Mannaerts, G.P. (1992) *J. Biol. Chem. 267*, 20065–20074.
94. Wanders, R.J.A., Denis, S., Jakobs, C., and ten Brink, H.J. (1992) *Progr. Clin. Biol. Res. 375*, 245–252.
95. Osumi, T., Ishii, N., Hijikata, M., Kamijo, K., Ozasa, H., Furuta, S., Miyazawa, S., Kondo, K., Inoue, K., Kagamiyama, H., and Hashimoto, T. (1985) *J. Biol. Chem. 260*, 8905–8910.
96. Hijikata, M., Wen, J.-K., Osumi, T., Hashimoto, T. (1990) *J. Biol. Chem. 265*, 4600–4606.
97. Lazo, O., Contreras, M., and Singh, I. (1990) *Biochemistry 29*, 3981–3986.
98. Lageweg, W., Tager, J.M., and Wanders, R.J.A. (1991) *Biochem. J. 276*, 53–56.
99. Singh, H., Beckman, K., and Poulos, A. (1993) *J. Biol. Chem. 269*, 9514–9520.
100. Lazarow, P.B. (1987) *J. Inherit. Metab. Dis. 10 (Suppl 1)*, 11–22.
101. Singh, I. (1992) *Progr. Clin. Biol. Res. 375*, 211–222.
102. Kaikaus, R.M., Ockner, R.K., and Bass, N.M. (1990) *Experientia 46*, 617–630.
103. Veerkamp, J.H., Peeters, R.A., and Maatman, R.G.H.J. (1991) *Biochim. Biophys. Acta 1081*, 1–24.
104. Bass, N.M., Manning, J.A., Ockner, R.K., Gordon, J.I., Seetharam, S., and Alpers, D.H. (1985) *J. Biol. Chem. 260*, 1432–1436.
105. Brandes, R., Kaikaus, R.M., Lysenko, N., Ockner, R.K., and Bass, N.M. (1990) *Biochim. Biophys. Acta 1034*, 53–61.
106. Ockner, R.K., Kaikaus, R.M., and Bass, N.M. (1992) *Progr. Clin. Biol. Res. 375*, 189–204.
107. Mosser, J., Douar, A.M., Sarde, C.O., Kioschis, P., Feil, R., Moser, H.W., Poustka, A.M., Mandel, J.M., and Aubourg, P. (1993) *Nature 361*, 726–730.
108. Cartier, N., Sarde, C.O., Douar, A.M., Mosser, J., Mandel, J.L., and Aubourg, P. (1993) *Hum. Mol. Genet. 2*, 1949–1951.
109. Higgins, C.F. (1992) *Ann. Rev. Cell Biol. 8*, 67–113.
110. Christensen, E., Gronn, M., Hagve, T.A., Kase, B.F., and Christophersen, B.O. (1989) *Biochim. Biophys. Acta 1002*, 79–83.
111. Christensen, E., Hagve, T.A., and Christophersen, B.O. (1988) *Biochim. Biophys. Acta 959*, 95–99.
112. Hashmi, M., Stanley, W., and Singh, I. (1986) *FEBS Lett. 86*, 247–250.
113. Laposata, M., Reich, E.L., and Majerus, P.W. (1985) *J. Biol. Chem. 260*, 11016–11020.
114. Herndon, J.H., Steinberg, D., Uhlendorf, B.W., and Fales, H.M. (1969) *J. Clin. Invest. 48*, 1017–1031.
115. Tsai, S.C., Avigan, J., and Steinberg, D. (1969) *J. Biol. Chem. 244*, 2682–2692.
116. Muralidharan, V.B., and Kishimoto, Y. (1984) *J. Biol. Chem. 259*, 13021–13026.
117. Steinberg, D. (1989) in *The Metabolic Basis of Inherited Disease, 6th Ed.* (Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., eds.), pp. 1533–1550. McGraw-Hill, New York.
118. Singh, I., Lazo, O., Kalipada, P., and Singh, A.K. (1993) *Biochim. Biophys. Acta 1180*, 221–224.
119. Poulos, A., Sharp, P., Singh, H., Johnson, D.W., Carey, W.F., and Easton, C.E. (1993) *Biochem. J. 292*, 457–461.
120. Kilponen, J.K., Palosaari, P.M., Sormunen, R.T., Vihinen, M., and Hiltunen, R.K. (1992) *Progr. Clin. Biol. Res. 375*, 33–40.
121. Palosaari, P.M., and Hiltunen, J.K. (1990) *J. Biol. Chem. 265*, 2446–2449.
122. Igarashi, M., Schaumburg, H.H., Powers, J., Kishimoto, Y., Kolodny, E., and Moser, H.W. (1976) *J. Neurochem. 26*, 851–860.
123. Igarashi, M., Belchis, D., and Suzuki, K. (1976) *J. Neurochem. 27*, 327–328.
124. Menkes, J.H., and Corbo, L.M. (1977) *Neurology 27*, 928–932.
125. Ramsey, R.B., Banik, N.L., and Davison, A.N. (1979) *J. Neurol. Sci. 40*, 189–196.
126. Molzer, B., Bernheimer, H., Budka, H., Pilz, B., and Toifl, K. (1981) *J. Neurol. Sci. 51*, 301–310.
127. Askanas, V., McLaughlin, J., Engel, W.K., and Adornato, B.T. (1979) *New Engl. J. Med. 301*, 588–590.
128. Moser, H.W., Moser, A.B., Kawamura, N., Murphy, J., Suzuki, K., Schaumburg, H.H., and Kishimoto, Y. (1980) *Ann. Neurol. 7*, 542–549.
129. Moser, H.W., Moser, A.B., Powers, J.M., Nitowsky, H.M., Schaumburg, H.H., Norum, R.A., and Migeon, B.R. (1982) *Pediatr. Res. 16*, 172–175.
130. Molzer, B., Bernheimer, H., Heller, R., Toifl, K., and Vetterlein, M. (1982) *Clin. Chim. Acta 125*, 299–305.
131. Theda, C., Moser, A.B., Powers, J.M., and Moser, H.W. (1992) *J. Neurol. Sci. 110*, 195–204.
132. Powers, J.M., Liu, Y., Moser, A.B., and Moser, H.W. (1992) *J. Neuropath. Exp. Neurol. 51*, 630–643.
133. Reinecke, C.J., Knoll, D.P., Pretorius, P.J., Steyn, H.S., and Simpson, R.H.W. (1985) *J. Neurol. Sci. 70*, 21–38.
134. Singh, I., Moser, A.B., Moser, H.W., and Kishimoto, Y. (1984) *Pediatr. Res. 18*, 286–290.
135. Singh, I., Moser, A.B., Goldfischer, S., and Moser, H.W. (1984) *Proc. Natl. Acad. Sci. USA 81*, 4203–4207.
136. Poulos, A., Singh, H., Paton, B., Sharp, P., and Derwas, N. (1986) *Clin. Genet. 29*, 397–408.
137. Wanders, R.J.A., van Roermund, C.W.J., van Wijland, M.J.A., Schutgens, R.B.H., Heikoop, J., van den Bosch, H., Schram, A.W., and Tager, J.M. (1987) *J. Clin. Invest. 80*, 1778–1783.
138. Lazo, O., Contreras, M., Hashmi, M., Stanley, W., Irazu, C., and Singh, I. (1988) *Proc. Natl. Acad. Sci. USA 85*, 7647–7651.
139. Wanders, R.J.A., van Roermund, C.W.T., van Wijland, M.J.A., Schutgens, R.B.H., van den Bosch, H., Schram, A.W., and Tager, J.M. (1988) *Biochem. Biophys. Res. Comm. 153*, 618–624.
140. Tsuji, S., Sano, T., Ariga, T., and Miyatake, T. (1981) *J. Biochem. (Tokyo) 90*, 1233–1236.
141. Tsuji, S., Ohno, T., Miyatake, T., Suzuki, A., and Yamakawa, T. (1984) *J. Biochem. (Tokyo) 96*, 1241–1247.
142. Tsuji, S., Sano-Kawamura, T., Ariga, T., and Miyatake, T. (1985) *J. Neurol. Sci. 71*, 359–367.
143. Koike, R., Tsuji, S., Ohno, T., Suzuki, Y., Orii, T., and Miyatake, T. (1991) *J. Neurol. Sci. 103*, 188–194.
144. Kishimoto, Y., Moser, H.W., Kawamura, N., Platt, M., Pallante, S.L., and Fenselau, C. (1980) *Biochem. Biophys. Res. Comm. 96*, 69–76.
145. Van Duyn, M.A., Moser, A.B., Brown, F.R., Sacktor, N., Liu, A., and Moser, H.W. (1984) *Am. J. Clin. Nutr. 40*, 277–284.
146. Brown, F.R., Van Duyn, M.A.S., Moser, A.B., Shulman, J.D., Rizzo, W.B., Snyder, R.D., Murphy, J.V., Kamoshita, S., Migeon, C.J., and Moser, H.W. (1982) *Johns Hopkins Med. J. 151*, 164–172.
147. Rizzo, W.B., Phillips, M.W., Dammann, A.L., Leshner, R.T., Avigan, J., and Proud, V.K. (1987) *Ann. Neurol. 21*, 232–239.
148. Moser, A.B., Borel, J., Odone, A., Naidu, S., Cornblath, D., Sanders, D.B., and Moser, H.W. (1987) *Ann. Neurol. 21*, 240–249.
149. Moser, H.W., Moser, A.B., Smith, K.D., Bergin, A., Borel, J., Shankroff, J., Stine, O.C., Merette, C., Ott, J., Krivit, W., and Shapiro, E. (1992) *J. Inher. Metab. Dis. 15*, 645–664.
150. Aubourg, P., Adamsbaum, C., Lavallard-Rousseau, M.C., Rocchiccioli, F., Cartier, N., Jambaque, I., Jakobezak, C., Lemaitre, A., Boureau, F., Wolf, C., and Bougneres, P.F. (1993) *New Engl. J. Med. 329*, 745–752.

151. Kaplan, P.W., Tusa, R.J., Shankroff, J., Heller, J.E., and Moser, H.W. (1993) *Ann. Neurol. 34*, 169–174.
152. Poulos, A., and Robertson, E.F. (1994) *Med. J. Aust. 160*, 315–317.
153. Poulos, A., Gibson, R., Sharp, P., Beckman, K., and Grattan-Smith, P. (1994) *Ann. Neurol. 36*, 741–746.
154. Rasmussen, M., Moser, A.B., Borel, J., Khangoora, S., and Moser, H.W. (1994) *Neurochem. Res.*, in press.
155. Poll-The, B.T., Roels, F., Ogier, H., Scotto, J., Vamecq, J., Schutgens, R.B.H., Wanders, R.J.A., van Roermund, C.W.T., Van Wijland, M.J.A., Schram, A.W., Tager, J.M., and Saudubray, J.-M. (1988) *Am. J. Hum. Genet. 42*, 422–434.
156. Wanders, R.J.A., Van Roermund, C.W.T., Schelen, A., Schutgens, R.B.H., Tager, J.M., Stephenson, J.B.P., and Clayton, P.T. (1990) *J. Inher. Metab. Dis. 13*, 375–379.
157. Watkins, P.A., Chen, W.W., Harris, C.J., Hoefler, G., Hoefler, S., Blake, D.C., Balfe, A., Kelley, R.I., Moser, A.B., Beard, M.E., and Moser H.W. (1989) *J. Clin. Invest. 83*, 771–777.
158. McGuinness, M.C., Moser, A.B., Poll-The, B.T., and Watkins, P.A. (1993) *Biochem. Med. Metab. Biol. 49*, 228–242.
159. Schram, A.W., Goldfischer, S., van Roermund, C.W.T., Brouwer-Kelder, E.M., Collins, J., Hashimoto, T., Heymans, H.S.A., van den Bosch, H., Schutgens, R.B.H., Tager, J.M., and Wanders, R.J.A. (1987) *Proc. Natl. Acad. Sci. USA 84*, 2494–2496.
160. Goldfischer, S., Moore, C.L., Johnson, A.B., Spiro, A.J., Valsamis, M.P., Wisniewski, H.K., Ritch, R.H., Norton, W.T., Rapin, I., and Gartner, L.M. (1973) *Science 182*, 62–64.
161. Jaffe, R., Crumrine, P., Hashida, Y., and Moser, H.W. (1982) *Am. J. Pathol. 108*, 100–111.
162. Poulos, A., Sharp, P., and Whiting, M. (1984) *Clin. Genet. 26*, 579–586.
163. Poulos, A., Sharp, P., Fellenberg, A.J., and Danks, D.M. (1985) *Hum. Genet. 70*, 172–177.
164. Poll-The, B.T., Saudubray, J.M., Ogier, H., Schutgens, R.B.H., Wanders, R.J.A., Schrakamp, G., van den Bosch, H., Trijbels, J., Poulos, A., Moser, H.W., van Elderen, J., and Eyssen, H.J. (1986) *J. Inherit. Dis. 9*, 169–174.
165. Wanders, R.J.A., Heymans, H.S.A., Schutgens, R.B.H., Barth, P.G., van den Bosch, H., and Tager, J.M. (1988) *J. Neurol. Sci. 88*, 1–39.
166. Hughes, J.L., Crane, D.I., Robertson, E., and Poulos, A. (1994) *Virchows Archiv. A Pathol. Anat. 423*, 459–468.
167. Moser, A.B., Singh, I., Brown, F.R., Solish, G.I., Kelley, R.I., Benke, P.J., and Moser, H.W. (1984) *New Engl. J. Med. 310*, 1141–1146.
168. Yajima, S., Suzuki, Y., Shimozawa, N., Yamaguchi, S., Orii, T., Fujiki, Y., Osumi, T., Hashimoto, T., and Moser, H.W. (1992) *Hum. Genet. 88*, 491–499.
169. Danks, D.M., Tippett, P., Adams, C., and Campbell, P. (1975) *J. Pediatr. 86*, 382–387.
170. Poulos, A., Pollard, A.C., Mitchell, J.D., Wise, G., and Mortimer, G. (1984) *Arch. Dis. Child. 59*, 222–229.
171. Budden, S.S., Kennaway, N.G., Buist, N.R.M., Poulos, A., and Weleber, R.G. (1986) *J. Pediatr. 108*, 33–39.
172. O'Brien, J.S., Fillerup, D.L., and Mead, J.F. (1964) *J. Lipid Res. 5*, 109–116.
173. O'Brien, J.S., and Sampson, E.L. (1965) *J. Lipid Res. 6*, 545–551.
174. Neuringer, M., Connor, W.E., Lin, D.S., Barstad, L., and Luck, S. (1986) *Proc. Natl. Acad. Sci. USA 83*, 4021–4025.
175. Sklar, L.A., Miljanich, G.P., Bursten, S.L., and Dratz, E.A. (1979) *J. Biol. Chem. 254*, 9583–9591.
176. Wu, C.S.C., Hachimori, A., and Yang, J.T. (1982) *Biochemistry 21*, 4556–4562.
177. Mooibroek, M.J., Cook, H.W., Clarke, J.T.R., and Spence, M.W. (1985) *J. Neurochem. 44*, 1551–1558.
178. Harzer, K., Paton, B.C., Poulos, A., Kustermann-Kuhn, B., Roggendorf, W., Grisar, T., and Popp, M. (1989) *Eur. J. Pediatr. 149*, 31–39.
179. Sugita, M., Dulaney, J.T., and Moser, H.W. (1972) *Science 178*, 1100–1102.
180. Tiffany, J.M. (1987) *Adv. Lipid Res. 22*, 1–62.
181. Tiffany, J.M., and Bron, A.J. (1978) *Trans. Ophthalmol. Soc. UK 98*, 335–338.
182. Selinger, D.S., Selinger, R.C., and Reed W.P. (1979) *Surv. Ophthalmol. 24*, 33–38.
183. Elias, P.M., and Friend, D.S. (1975) *J. Cell Biol. 65*, 180–191.
184. Prottey, C., Hartop, P.J., and Press, M. (1975) *J. Invest. Dermatol. 64*, 228–234.
185. Houtsmuller, U.M.T., and van der Beek, A. (1982) *Progr. Lipid Res. 20*, 219–224.
186. Horie, S., Suzuki, T., and Suga, T. (1989) *Arch. Biochem. Biophys. 274*, 64–73.
187. Hovik, R., Brodal, B., Bartlett, K., and Osmundsen, H. (1991) *J. Lipid Res. 32*, 993–999.
188. Thompson, S.L., Burrow, R., Laub, R.J., and Krisans, S.K. (1987) *J. Biol. Chem. 262*, 17420–17425.
189. Scallen, T.J., Noland, B.J., Gavey, K.L., Bass, N.M., Ockner, R.K., Chanderbahn, R., and Vahouny, G.V. (1985) *J. Biol. Chem. 260*, 4733–4739.
190. Seltman, H., Diven, W., Rizk, M., Noland, B.J., Chanderbahn, R., Scallen, T.J., Vahouny, G., and Sanghvi, A. (1985) *Biochem. J. 230*, 19–24.
191. Keller, G.A., Pazirandeh, M., and Krisans, S. (1986) *J. Cell Biol. 103*, 875–886.
192. Whitcomb, R.W., Linehan, W.M., and Knazek, R.A. (1988) *J. Clin. Invest. 81*, 185–188.
193. Martinez, M. (1989) *Lipids 24*, 261–265.
194. Martinez, M. (1990) *Neurology 40*, 1292–1298.
195. Mathis, R.K., Watkins, J.B., Szczpanik-Van Leeuween, P., and Lott, I. (1980) *Gastroenterology 79*, 1311–1317.
196. Hughes, J.L., Poulos, A., Robertson, E.F., Chow, C.W., Sheffield, L.J., Christodoulou, J., and Carter, R.F. (1990) *Virchows Archiv. A. Pathol. Anat. 416*, 255–264.
197. Miki, H., Matsuzawa, Y., Ishibe, T., Ishikawa, K., Moriwaki, K., Takahashi, M., Tsumori, T., Yamamoto, A., and Tarui, S. (1983) *J. Neurol. 229*, 1–10.
198. Van Rollins, M., Baker, R.C., Sprecher, H.W., and Murphy, R.C. (1984) *J. Biol. Chem. 259*, 5776–5783.
199. Samuelsson, B., Dahlen, S.E., Lindgren, J.A., Rouzer, C.A., and Serhan, C.N. (1987) *Science 237*, 1171–1176.
200. Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., and Lefkowith, J.B. (1986) *Ann. Rev. Biochem. 55*, 69–102.
201. Poulos, A., Aveldano, M., Ferrante, A., Hardy, S.J., Johnson, D.W., Robinson, B.S., Sharp, P., and Singh, H. (1993) in *Essential Fatty Acids and Eicosanoids: Invited Papers from the Third International Congress* (Sinclair, A.J., and Gibson, R.A., eds.), pp. 45–48, AOCS Press, Champaign.
202. Nishizuka, Y. (1986) *Science 233*, 305–312.
203. Majerus, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T.E., Ishii, H., Bansal, V.S., and Wilson, D.B. (1986) *Science 234*, 1519–1526.
204. Huang, C., and Cabot, M.C. (1990) *J. Biol. Chem. 265*, 14858–14863.
205. Billah, M.M., and Anthes, J.C. (1990) *Biochem. J. 269*, 281–291.
206. Pelech, S.L, and Vance, D.E. (1989) *TIBS 14*, 28–30.
207. Kiss, Z., and Anderson, W.B. (1989) *J. Biol. Chem.* 264, 1483–1487.
208. Kiss, Z., and Anderson, W.B. (1989) *FEBS Lett. 257*, 45–48.
209. Hii, C.S.T., Edwards, Y.S., and Murray, A.W. (1991) *J. Biol. Chem. 266*, 20238–20243.
210. Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W., and Siegel, M.I. (1989) *J. Biol. Chem. 264*, 17069–17077.

211. Mullmann, T.J., Siegel, M.I., Egan, R.W., and Billah, M.M. (1990) *Biochem. Biophys. Res. Comm. 170*, 1197–1202.
212. Perry, D.K., Stevens, V.L., Widlanski, T.S., and Lambeth, J.D. (1993) *J. Biol. Chem. 268*, 25302–25310.
213. Jalink, K., van Corven, E.J., and Moolenaar, W.H. (1990) *J. Biol. Chem. 265*, 12232–12239.
214. Agwu, D.E., McPhail, L.C., Sozzani, S., Bass, D.A., and McCall, C.E. (1991) *J. Clin. Invest. 88*, 531–539.
215. Hannun, Y.A., and Linardic, C.M. (1993) *Biochim. Biophys. Acta 1154*, 223–236.
216. Okazaki, T., Bielawska, A, Bell, R.M., and Hannun, Y. (1990) *J. Biol. Chem. 265*, 15823–15831.
217. Kim, M.Y., Linardic, C., Obeid, L., and Hannun, Y.A. (1991) *J. Biol. Chem. 266*, 484–489.
218. Mathias, S., Younes, A., Kan, C.C., Orlow, I., Joseph, C., and Kolesnick, R.N. (1993) *Science 259*, 519–522.
219. Dobrowsky, R.T., and Hannun, A.Y. (1992) *J. Biol. Chem. 267*, 5048–5051.
220. Dobrowsky, R.T., Kamibayashi, C., Mumby, M.C., and Hannun, A.Y. (1993) *J. Biol. Chem. 268*, 15523–155.
221. Mathias, S., Dressler, K.A., and Kolesnick, R.N. (1991) *Proc. Natl. Acad. Sci. USA 88*, 10009–10013.
222. Clark, M.A., Chen, M.J., Crooke, S.T., and Bomalaski, J.S. (1988) *Biochem. J. 250*, 125–132.
223. Walker, J.M., and Sando, J.J. (1988) *J. Biol. Chem. 263*, 4537–4540.
224. Bazzi, M.D., and Nelsestuen, G.L. (1987) *Biochemistry 26*, 115–122.
225. Badwey, J.A., Curnutte, J.T., Robinson, J.M., Berde, C.B., Karnovsky, M.J., and Karnovsky, M.L. (1984) *J. Biol. Chem. 259*, 7870–7877.
226. Poulos, A., Robinson, B.S., Ferrante, A., Harvey, D.P., Hardy, S.J., and Murray, A.W. (1991) *Immunology 73*, 102–108.
227. Hardy, S.J., Robinson, B.S., Poulos, A., Harvey, D.P., Ferrante, A., and Murray, A.W. (1991) *Eur. J. Biochem. 198*, 801–806.
228. Bromberg, Y., and Pick, E. (1984) *Cell Immunol. 88*, 213–221.
229. Ferrante, A., Goh, D., Harvey, D.P., Robinson, B.S., Bates, E.J., and Poulos, A. (1994) *J. Clin. Invest. 93*, 1063–1070.
230. Bates, E.J., Ferrante, A., Harvey, D.P., and Poulos, A. (1993) *J. Leuk. Biol. 53*, 420–426.
231. McPhail, L.C., Clayton, C.C., and Synderman, R. (1984) *Science 224*, 622–625.
232. Hardy, S., Ferrante, A., Poulos, A., Robinson, B.S., Johnson, B.S., and Murray, A.W. (1994) *J. Immunol. 153,* 1754–1761.
233. Hardy, S.J., Ferrante, A., Robinson, B.S., Johnson, D.W., Poulos, A., Clark, K.J., and Murray, A.W. (1994) *J. Neurochem. 62*, 1546–1551.
234. Singh, H., and Poulos, A. (1986) *Arch. Biochem. Biophys. 250*, 171–179.
235. Kling, M.R., Easton, C.J., and Poulos, A. (1993) *J. Chem. Soc. Perkin Trans. 1*, 1183–1189.
236. Johnson, D.W., (1990) *Chem. Phys. Lipids 56*, 65–71.
237. Griffin, D.E., Moser, H.W., Mendoza, Q., Moench, T., O'Toole, S., and Moser, A.B. (1985) *Ann. Neurol. 18*, 660–664.
238. Shimozawa, N., Tsukamoto, T., Suzuki, Y., Orii, T., Shirayoshi, Y., Mori, T., and Fujiki, Y. (1992) *Science 255*, 1132–1134.
239. Gartner, J., Moser, H.W., and Valle, D. (1992) *Nature Genet. 1*, 16–23.

[Received June 30, 1994, and in revised form November 23, 1994; Revision accepted December 8, 1994]

ARTICLE

Trans Fatty Acid Isomers in Canadian Human Milk

Z.-Y. Chen*, G. Pelletier, R. Hollywood and W.M.N. Ratnayake

Nutrition Research Division, Food Directorate, Health Protection Branch, Health Canada, Ottawa, Ontario, K1A 0L2 Canada

ABSTRACT: The fatty acid composition, total *trans* content (i.e., sum of all the fatty acids which may have one or more *trans* double bonds) and geometric and positional isomer distribution of unsaturated fatty acids of 198 human milk samples collected in 1992 from nine provinces of Canada were determined using a combination of capillary gas–liquid chromatography and silver nitrate thin-layer chromatography. The mean total *trans* fatty acid content was 7.19 ± 3.03% of the total milk fatty acids and ranged from 0.10 to 17.15%. Twenty-five of the 198 samples contained more than 10% total *trans* fatty acids, and thirteen samples contained less than 4%. Total *trans* isomers of linoleic acid were 0.89% of the total milk fatty acids with 18:2Δ9*c*,13*t* being the most prevalent isomer, followed by 18:2Δ9*c*,12*t* and 18:2Δ9*t*,12*c*. Using the total *trans* values in human milk determined in the present study, the intake of total *trans* fatty acids from various dietary sources by Canadian lactating women was estimated to be 10.6 ± 3.7 g/person/d, and in some individuals, the intake could be as high as 20.3 g/d. The 18:1 *trans* isomer distribution differed from that of cow's milk fat but was remarkably similar to that in partially hydrogenated soybean and canola oils, suggesting that partially hydrogenated vegetable oils are the major source of these *trans* fatty acids. *Lipids 30,* 15–21 (1995).

Partially hydrogenated fats contain an assortment of geometric and positional isomers of unsaturated fatty acids, of which the *trans* isomers have been reported to raise serum low-density lipoprotein cholesterol (1,2) and lower high-density lipoprotein cholesterol (1). Recent epidemiological and clinical studies have suggested that intake of *trans* fatty acids may be a risk factor in cardiovascular disease (3,4). Some other reports, however, have indicated no such health risk associated with *trans* fatty acids (5,6). *Trans* fatty acids may also interfere with biosynthesis of long-chain polyunsaturated fatty acids during development (7–10). In essential fatty acid deficiency, some of the isomeric fatty acids, particularly the mono-*trans* geometrical isomers of linoleic acid (18:2n-6), are desaturated and elongated to produce unusual longer chain polyunsaturated fatty acid metabolites in rodents (10–12). The major *trans* isomer of 18:2n-6 in partially hydrogenated vegetable oils is 18:2Δ9*c*,13*t* (13,14). Recently, we have shown that 18:2Δ9*c*,13*t* and three other isomeric fatty acids in partially hydrogenated vegetable oils, *viz.*, 18:Δ12*c*, 18:2Δ9*c*,12*t* and 18:2Δ9*t*,12*c*, are metabolized to unusual C_{20} polyunsaturated fatty acid isomers even under sufficient intake of 18:2n-6 (1.46% energy; Ref. 15). *Trans* fatty acids have been reported to occur in human milk (16–19). Distribution of *trans* and *cis* isomers has been determined in human tissues (20), but similar data are not yet available for human milk. These *trans* isomers must be of dietary origin, because they cannot be synthesized by mammals. Dietary *trans* fatty acids have also been shown to depress the milk fat content in mice regardless of total fat intake or the dietary level of 18:2n-6 (21,22), but not in swine (23).

To determine the relative contribution of *trans* fatty acid isomers from dietary sources, the present study compared the isomer distribution profile in human milk fat with that in partially hydrogenated vegetable oils and also that in cow's milk. We also sought to ascertain whether dietary *trans* fatty acids inhibit human milk fat production. This was done by correlating the milk fat content with total milk *trans* fatty acid content, which is defined as the sum of all the fatty acids with one or more double *trans* double bonds. Furthermore, *trans* fatty acid consumption of Canadian lactating women was estimated based on the *trans* fatty acid content in their milk.

MATERIALS AND METHODS

Materials. The human milk samples used in this study were from the 1992 collection of Health Protection Branch's ongoing monitoring program of chlorinated hydrocarbon contaminants in the breast milk of Canadian women (unpublished work of Dr. W.H. Newsome, Health Protection Branch, Ottawa, Canada). Samples of "mature milk" (3 to 4 wk after parturition) were collected from donors from nine provinces of Canada (20–25 samples/province) who had resided in Canada for at least 5 yr. Donors were requested to express their milk manually several times during each feeding, alternating between right and left breast. The sample from each donor thus represented the accumulated milk collection of one day. A total of 198 samples was collected in residue-free bottles shipped and stored as reported earlier (24).

*To whom correspondence should be addressed at Department of Biochemistry, The Chinese University of Hong Kong, Shatin, NT, Hong Kong.

Abbreviations: $AgNO_3$–TLC, silver nitrate thin-layer chromatography; *c, cis*; FAME, fatty acid methyl esters; GLC, gas–liquid chromatography; HTM, high-*trans* milk; LTM, low-*trans* milk; MTM, medium-*trans* milk; PHCO, partially hydrogenated canola oil; PHSO, partially hydrogenated soybean oil; *t, trans*.

Copyright © 1995 by AOCS Press

Twelve basestocks. Partially hydrogenated canola (PHCO) and soybean (PHSO) basestocks with different contents of *trans* fatty acids and iodine values were supplied by CanAmera Foods, Inc. (Toronto, Ontario, Canada), which is one of the largest edible oil processors. It also supplies their basestocks to most of the major food chains in Canada. We therefore believe that the basestocks analyzed are representative of what are in the Canadian market. Homogenized cow's milk was purchased locally.

Milk lipid extraction. Milk samples were thawed at room temperature (25°C). Fat from a 5 g sample was extracted using 25 vol of $CHCl_3$/MeOH (2:1, vol/vol) containing 0.02% butylated hydroxytoluene (Sigma Chemicals, St. Louis, MO) as an antioxidant and triheptadecanoin (1 mg/mL; Sigma Chemicals) as an internal standard to quantitate total milk fat (25). Another 5 g sample was extracted with the same solvent system but without addition of triheptadecanoin to determine the content of endogenous heptadecanoic acid in milk. Cow's milk fat was similarly extracted.

Fatty acid analysis. The extracted lipids were converted to fatty acid methyl esters (FAME) using 14% BF_3/MeOH reagent (Sigma Chemicals) at 90°C for 50 min under nitrogen. FAME were analyzed by gas–liquid chromatography (GLC) using an SP-2560 flexible fused silica capillary column (100 m × 0.25 mm i.d., 20 μm film thickness; Supelco, Inc. Bellefonte, PA) in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame-ionization detector (Palo Alto, CA). Column temperature was programmed from 150 to 180°C at a rate of 0.5°C/min, and then to 210°C at a rate of 3°C/min. Injector and detector temperatures were 250 and 300°C, respectively. Hydrogen was used as the carrier gas at a head pressure of 20 psi.

The gas chromatographic trace of the FAME profile of human milk is shown in Figure 1. Single step, direct GLC cannot determine the total 18:1 *trans* due to overlap of high delta 18:1 *trans* isomers (18:1Δ12*t* to 18:1Δ16*t*) with 18:1 *cis* isomer peaks (26). We found that an average of 20.8% of total 18:1 *trans* were actually overlapped with 18:1 *cis* fraction. The extent of overlap primarily was dependent on the total *trans* content and ranged from 9–30%. The higher the total 18:1 *trans*, the greater the overlap. Therefore, the total 18:1 *trans* and total 18:1 *cis* contents in the milk samples were de-

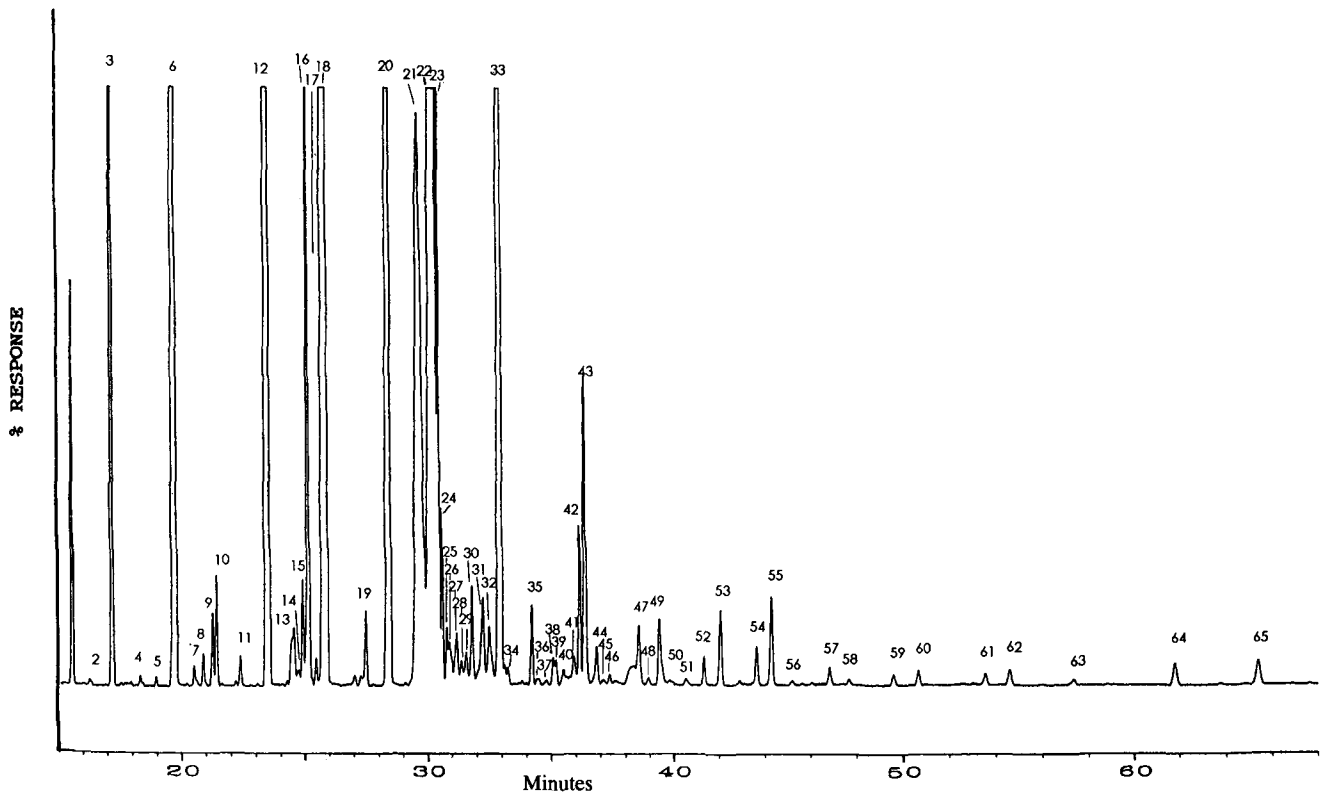


FIG. 1. A typical chromatogram of the C_{10} to C_{22} region of fatty acid methyl esters from a sample of Canadian human milk total lipids on an SP-2560 capillary column (100 m × 0.25 mm). Peaks were identified as: 1, 10:0; 2, 11:0; 3, 12:0; 4, 13:0; 5, 14:0 *br*; 6, 14:0; 7, 14:1Δ9*t*; 8, 14:1Δ7*c*; 9, 14:1Δ9*c*; 10, 15:0; 11, 16:0 *br* + 15:1 ?; 12, 16:0; 13, 16:1Δ7*t*; 14, 16:1Δ9t; 15, 16:1Δ7*c*; 16, 16:1Δ9*c*; 17, unknown; 18, 17:0 (internal standard); 19, 17:1Δ10*c*; 20, 18:0; 21, 18:1(Δ7–Δ11)*t*; 22, 18:1(Δ7–Δ10)*c* + 18:1(Δ12–Δ15)*t*; 23, 18:1Δ11*c*; 24, 18:1Δ12*c*; 25, 18:1Δ13*c*; 26, 18:2*tt*; 27, 18:1Δ14*c* + 18:1Δ16*t*; 28, 18:1Δ15*c*; 29, 18:1Δ16*c* + 18:2Δ9*t*,12*t*; 30, 18:2Δ9*c*,13*t* + 18:2Δ8*t*,12*c*; 31, 18:2Δ9*c*,12*t*; 32, 18:2Δ9*t*,12*c*; 33, 18:2n-6; 34, 18:2Δ9*c*,15*c*; 35, 20:0; 36, unknown; 37, unknown; 38, 18:3n-6; 39 + 40 + 41, 18:3*t*; 42, 20:1Δ11*c*; 43, 18:3n-3; 44, 20:1Δ13*c*; 45+46, 18:2 conjugates; 47, 20:2Δ8*c*,14*c*; 48, 18:2 conjugate; 49, 20:2n-6; 50, 18:2 conjugate; 51, 20:3Δ5*c*,8*c*,11*c*; 52, 20:3Δ5*c*,11*c*,14*c*; 53, 20:3n-6; 54, 22:1Δ13*c*; 55, 20:4n-6; 56, 20:4n-3; 57, unknown; 58, unknown; 59, 20:5n-3; 60, 24:0; 61, 24:1*c*; 62, 22:4n-6; 63, 22:5n-6; 64, 22:5n-3; 65, 22:6n-3.

termined using silver nitrate thin-layer chromatography ($AgNO_3$–TLC) in conjunction with capillary GLC (26). Preparative $AgNO_3$–TLC plates were prepared as described previously (26). FAME (~15 mg) were applied to the plates in hexane solution using a TLC streaker (Applied Science Laboratories, State College, PA). The plates were developed in toluene at –20°C for 3 h. The separated bands were visualized under ultraviolet light (254 nm) after spraying with 0.2% dichlorofluorescein in ethanol. The 18:1 *trans* band was scraped off the plate and extracted with hexane/$CHCl_3$ (1:1, vol/vol). After drying under nitrogen, the 18:1 *trans* fraction was redissolved in hexane, and analyzed by GLC using an SP-2560 capillary column. The proportion of 18:1 *trans* isomers (the high delta value *trans* isomers) that overlapped with the 18:1 *cis* isomer peaks were calculated by comparing the 18:1 region of the GLC chromatogram of the isolated 18:1 *trans* fraction with that of the parent FAME mixture prior to $AgNO_3$–TLC fractionation. For this purpose, the 18:1 *trans* isomer peaks (18:1Δ7*t* to 18:1Δ11*t*) that were well separated from the *cis* peaks served as the internal standard. The total 18:1 *trans* content was then calculated, by summing up the proportions of the 18:1 *trans* isomers (18:1Δ12*t* to 18:1Δ16*t*) that overlapped with the *cis* isomers and the well separated 18:1 *trans* isomers.

The distribution of 18:1 *trans* and *cis* isomers isolated by $AgNO_3$–TLC was determined by BF_3/MeOH oxidative ozonolysis (27). Ozone was generated in a Model T-408 Polymetrics Laboratory Ozonator (Polymetrics, Inc., San Jose, CA). Calculation of individual monounsaturated isomers was based on the proportion of the corresponding dimethyl ester products. The dimethyl esters were identified according to Ratnayake and Pelletier (14).

The geometric and positional isomers of 18:2n-6 in human milk were identified and determined as described previously (14).

Statistics. Data were expressed as mean ± standard deviation. Analysis of variance followed by Student's *t*-test (two-tailed) where applicable was used for statistical evaluation of significant difference between groups.

RESULTS

The fatty acid data of Canadian breast milk are given in Tables 1 and 2. The mean fat content of the samples, calculated using triheptadecanoin as the internal standard (25), was 31.6 ± 9.4 g/L. A correlation between the fat content and percentage of total *trans* fatty acids was not observed; however, 18:2n-6 and α-linolenic acid (18:3n-3) were inversely related to the total *trans* fatty acids and 18:1 *trans* isomers (Fig. 2). In addition, total 18:1 *trans* isomers were positively correlated to stearic acid (18:0; $P < 0.01$) but it was negatively correlated with oleic acid (18:1n-9; $P < 0.05$; data not shown). The saturated fatty acids comprised 38.5% total milk fatty acids, approximately half of which was palmitic acid (16:0). The second major group was n-9 monounsaturated fatty acids, of which oleic acid (18:1Δ9) contributed the most (30.7%); 18:2n-6 varied

TABLE 1
Fatty Acid Composition (wt% total fatty acids) of Canadian Human Milk Determined by GC and Ag–TLC Plus GC[a]

Fatty acids	Direct GC	Ag–TLC/GC	Range
Saturated fatty acids			
10:0	1.39(0.59)	1.39(0.59)	0.46–4.42
11:0	0.01(0.02)	0.01(0.02)	trace–0.08
12:0	5.68(2.01)	5.68(2.01)	2.32–11.77
13:0	0.03(0.03)	0.03(0.03)	trace–0.14
14:0	6.10(1.73)	6.10(1.73)	2.26–11.68
15:0	0.37(0.12)	0.37(0.12)	0.12–0.67
16:0	18.30(2.25)	18.30(2.25)	12.90–24.06
17:0	0.32(0.08)	0.32(0.08)	0.03–0.44
18:0	6.15(0.97)	6.15(0.97)	3.49–9.85
20:0	0.15(0.09)	0.15(0.09)	trace–0.36
14:0 *br*	0.14(0.06)	0.14(0.06)	trace–0.26
16:0 *br*	0.14(0.06)	0.14(0.06)	0.04–0.45
Cis-monounsaturated fatty acids			
14:1Δ9*c*	0.28(0.08)	0.28(0.08)	0.06–0.66
16:1Δ9*c*	2.27(0.56)	2.27(0.56)	1.11–3.88
16:1Δ7*c*	0.41(0.13)	0.41(0.13)	0.20–0.70
17:1Δ10*c*	0.21(0.06)	0.21(0.06)	trace–0.44
18:1Δ9*c*	32.66(3.22)	30.65(2.66)	23.55–40.64
18:1Δ11*c*	1.91(0.17)	1.91(0.17)	1.26–2.33
Total other 18:1*c*	1.52(0.12)	2.31(0.23)	1.44–3.97
20:1Δ11*c*	0.39(0.13)	0.39(0.13)	0.13–0.65
20:1Δ13*c*	0.14(0.09)	0.14(0.09)	trace–0.42
22:1Δ13*c*	0.02(0.03)	0.02(0.03)	trace–0.11
n-6 Polyunsaturated fatty acids			
18:2n-6	10.47(2.62)	10.47(2.62)	5.79–21.35
18:3n-6	0.08(0.06)	0.08(0.06)	trace–0.21
20:2n-6	0.17(0.37)	0.17(0.09)	trace–0.35
20:3n-6	0.26(0.09)	0.26(0.09)	trace–0.46
20:4n-6	0.35(0.11)	0.35(0.11)	0.05–0.69
22:4n-6	0.04(0.05)	0.04(0.05)	trace–0.18
22:5n-6	0.01(0.02)	0.02(0.02)	trace–0.16
n-3 Polyunsaturated fatty acids			
18:3n-3	1.16(0.37)	1.16(0.37)	0.58–1.90
20:4n-3	0.06(0.06)	0.06(0.076)	trace–0.26
20:5n-3	0.05(0.05)	0.05(0.05)	trace–0.25
22:5n-3	0.08(0.06)	0.08(0.06)	trace–0.45
22:6n-3	0.14(0.10)	0.14(0.10)	trace–0.53
Trans fatty acids			
14:1Δ7*t*	0.09(0.05)	0.09(0.05)	trace–0.47
16:1Δ9*t*	0.18(0.08)	0.18(0.08)	trace–0.42
Total 18:1*t*	4.65(1.99)	5.87(2.52)	0.10–15.42
18:2Δ9*t*,12*t*	0.05(0.08)	0.05(0.08)	trace–0.28
18:2Δ9*c*,13*t*/8*t*,12*c*	0.36(0.14)	0.36(0.14)	trace–0.76
18:2Δ9*c*,12*t*	0.29(0.15)	0.29(0.15)	trace–0.78
18:2Δ9*t*,12*c*	0.24(0.12)	0.24(0.12)	trace–0.59
Total 18:3 *trans*	0.11(0.08)	0.11(0.08)	trace–0.34
Unusual *cis*–polyunsaturated fatty acids			
18:2Δ9*c*,15*c*	0.07(0.05)	0.07(0.05)	trace–0.15
20:2Δ8*c*,14*c*	0.19(0.08)	0.19(0.08)	trace–0.49
20:3Δ5*c*,11*c*,14*c*	0.02(0.02)	0.02(0.02)	trace–0.12
20:3Δ5*c*,8*c*,11*c*	0.05(0.06)	0.05(0.06)	trace–0.18
Others	2.24(0.09)	2.24(0.09)	1.25–2.50

[a]Data are mean (SD); n = 198. GC, gas chromatography; Ag–TLC, silver thin-layer chromatography; *br* = branched; Other 18:1*c* = 18:1*c* isomers other than 18:1Δ9*c*; trace = trace amount (<0.005% of total fatty acids); Others = this group includes fatty acids <10:0, very minor branched-chain fatty acids, 18:2 conjugated fatty acids and unknowns.

TABLE 2
Total Content (g/L) and Fatty Acid Composition (wt% of total fatty acids) of Canadian Human Milk Determined by Ag–TLC Plus GC[a]

Fatty acid class	% of total fatty acids
Total saturated fatty acids	38.50 (2.94)
Total C_{10}–C_{13} saturated fatty acids	7.11 (2.53)
Total C_{14-16} saturated fatty acids	24.77 (3.26)
Total n-6 fatty acids	11.39 (3.18)
Total n-3 fatty acids	1.49 (0.44)
Total n-6 long-chain fatty acids	0.83 (0.28)
Total n-3 long-chain fatty acids	0.33 (0.24)
Total *trans* fatty acids	7.19 (3.03)
Total 18:1 *trans* fatty acids	5.87 (2.52)
Total 18:1 *cis* other than 18:1n-9	4.22 (0.96)
Total milk fat (g/L)	31.58 (9.37)

[a]Data are mean (SD); n = 198. Abbreviations as in Table 1.

from 5.8 to 21.4% with an average value of 10.5%; 18:3n-3 accounted for 1.2% total milk fatty acids with a range of 0.6 to 1.9%. The longer-chain products of 18:2n-6 and 18:3n-3 represented 0.8 and 0.3% of total fatty acids, respectively. A correlation between both 18:2n-6 and 18:3n-3 and their corresponding long-chain metabolites was not observed.

Trans fatty acids were found in all the samples and ranged from 0.1 to 17.2% with a mean of 7.2 ± 3.0%. Twenty-five out of the 198 samples contained more than 10% total *trans* fatty acids. Furthermore, more than 15% *trans* fatty acids was found in 2% of the samples.

As expected, 18:1 *trans* was the major *trans* isomer group. It ranged from 0.1 to 15.4% of total fatty acids with a mean value of 5.9%. The average 18:1 *trans* isomer distribution is illustrated in Figure 3. In addition, the average 18:1 *trans* isomer distributions for PHSO, PHCO and cow's milk fat are also presented in Figure 3 for comparison. In the human milk samples analyzed, the most prevalent isomer was Δ11*t*, followed by Δ10*t*, Δ9*t*, Δ12*t* and Δ13*t* in a decreasing order. The 18:1 *trans* isomer distribution closely resembled that of PHSO, and to a lesser extent to the pattern in PHCO, but differed from that of cow's milk fat. In contrast to human milk fat and partially hydrogenated vegetable oils, cow's milk fat was characterized by having as much as 43% of total 18:1 *trans* isomers represented by 18:1Δ11*t* and low proportions of other isomers.

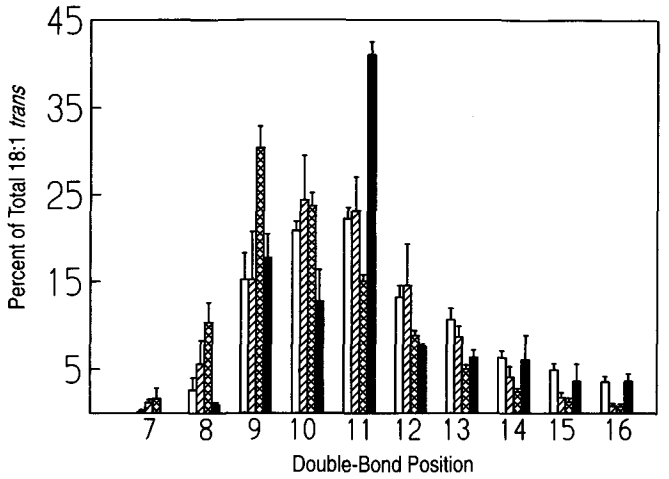


FIG. 3. Distribution of individual 18:1 *trans* isomer in human milk lipids (open bar, n = 198), partially hydrogenated soybean oil (hatched bar, n = 6), partially hydrogenated canola oil (cross-hatched bar, n = 6) and in cow's milk fat (solid bar, n = 6). Data are mean ± standard deviation. Total 18:1 *trans* isomers (wt% of total fatty acids) were 5.87% in human milk, 31.17% in partially hydrogenated soybean, 32.51% in partially hydrogenated canola oil and 2.10% in cow's milk fat.

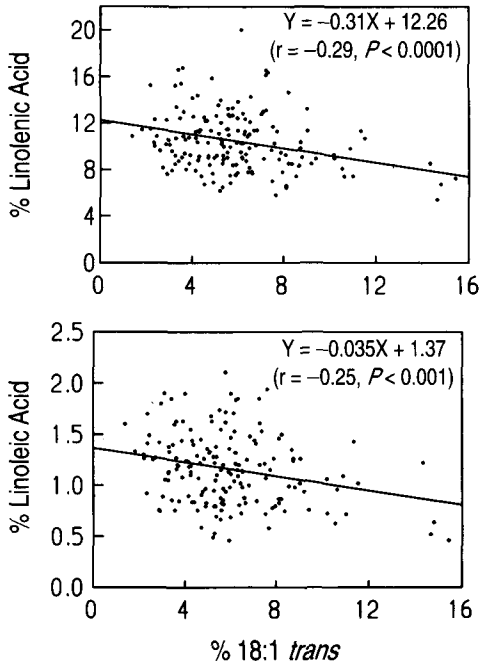


FIG. 2. Correlation of percent 18:2n-6 and percent 18:3n-3 with percent 18:1 *trans* in human milk lipids (n = 198).

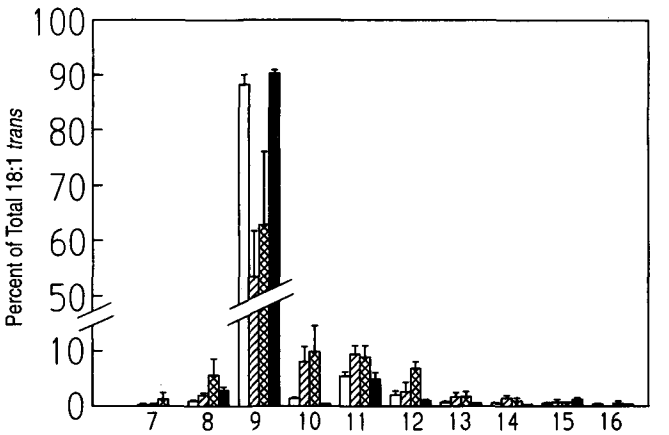


FIG. 4. Distribution of individual 18:1 *cis* isomer in human milk lipids (open bar, n = 198), in partially hydrogenated soybean oil (hatched bar, n = 6), in partially hydrogenated canola oil (cross-hatched bar, n = 6) and in cow's milk fat (solid bar, n = 6). Data are mean ± standard deviation. Total 18:1 *cis* isomers (wt% of total fatty acids) were 36.88% in human milk, 38.04% in partially hydrogenated soybean, 44.19% in partially hydrogenated canola oil and 22.91% in cow's milk fat.

TABLE 3
Fatty Acid Composition (wt%) of High *Trans* Milk, Low *Trans* Milk and Medium *Trans* Milk Determined by Ag–TLC Plus GC[a]

Fatty acid	High *trans* (n = 25)	Medium *trans* (n = 155)	Low *trans* (n = 18)
18:2n-6	9.12 ± 1.96[a]	10.50 ± 2.51[a,b]	11.70 ± 2.51[b]
18:3n-3	0.97 ± 0.27[a]	1.19 ± 0.36[a,b]	1.28 ± 0.32[b]
18:1*t*	10.35 ± 2.21[a]	5.42 ± 1.32[b]	2.64 ± 1.18[c]
18:1Δ9*c*	29.41 ± 3.47	30.75 ± 3.66	30.52 ± 3.59
Total other 18:1*c*	3.93 ± 0.46	4.24 ± 0.49	4.08 ± 0.48
18:2Δ9*c*,13*t*/8*t*,12*c*	0.51 ± 0.14[a]	0.36 ± 0.13[a]	0.13 ± 0.11[b]
18:2Δ9*c*,12*t*	0.42 ± 0.15[a]	0.28 ± 0.13[a]	0.12 ± 0.11[b]
18:2Δ9*t*,12*c*	0.37 ± 0.11[a]	0.24 ± 0.12[a]	0.07 ± 0.07[b]
20:4n-6	0.33 ± 0.06	0.35 ± 0.13	0.36 ± 0.12
22:6n-3	0.13 ± 0.06	0.14 ± 0.12	0.16 ± 0.07
Total *trans*	12.40 ± 2.41[a]	6.92 ± 1.50[b]	3.10 ± 0.97[c]

[a]Data are mean ± SD. Means in the same row with different superscripts (a, b, c) differ, $P < 0.01$. Total other 18:1*c* is the sum of 18:1 *cis* isomers excluding 18:1Δ9*c*. Abbreviations as in Table 1.

The distribution patterns of 18:1 *cis* isomers in human milk fat, partially hydrogenated vegetable oil and cow's milk fat were not comparable (Fig. 4). In human milk, the total 18:1 *cis* isomer group was largely oleic acid (Δ9*c*; 88.2%), followed by *cis*-vaccenic acid (Δ11*c*; 5.5%) with a small amount of other isomers ranging from Δ8 to Δ16. This is in contrast to PHSO and PHCO, where 18:1Δ9*c* accounted for 54–63% of total 18:1 *cis* isomers followed by Δ12*c* (7–18%), Δ11*c* (8–9%) and Δ10*c* (7–8%). In cow's milk, the Δ9*c* isomer represented 90% of the total 18:1 *cis* isomers with Δ11*c* and Δ8*c* being less than 5 and 3%, respectively (Fig. 4).

Trans isomers of 18:2n-6 amounted to 0.9% of total milk fatty acids (Table 1). This group primarily was composed of four isomers, 18:2Δ9*c*,13*t*/8*t*,12*t* (0.4%), 18:2Δ9*c*,12*t* (0.3%) and 18:2Δ9*t*,12*c* (0.2%). In the partially hydrogenated vegetable oils analyzed in the present study, these isomers accounted for 2.0, 1.1 and 1.0% of total fatty acids, respectively. The 18:2Δ9*c*,13*t* and 18:2Δ8*t*,12*c* are not separable by GLC or silver TLC, and hence are listed together (Tables 1 and 3). Of these two isomers, however, 18:1Δ9*c*,13*t* is the major one in PHSO and PHCO; and 18:2Δ8*t*,12*c* normally presents at extremely low level (14).

DISCUSSION

Significant regional differences in the average fatty acid composition data of Canadian human milk samples were not observed in this study (data not shown). However, within this population there were wide variations in the content of *trans* fatty acid in breast milk. Thus, we have reclassified the human milk samples into three categories (Table 3) according to the content of *trans* fatty acids; high-*trans* milk (HTM) for samples containing >10% *trans*, medium-*trans* milk (MTM) for samples containing ≥4% but ≤10% *trans*, and low-*trans* milk (LTM) for samples containing <4% *trans*. The high content of *trans* fatty acids in HTM was associated with lower levels of 18:2n-6 and 18:3n-3 than in MTM and LTM (Fig. 2; Table 3).

The total number of HTM accounted for 12% of total samples analyzed, and MTM accounted for 78%. This may indicate that the diet of a very large (~90%) segment of Canadian lactating women contains moderate to high levels of *trans* fatty acids. The fatty acid composition of milk from lactating women reflects to a certain extent the fatty acid composition of diets from previous days. This is particularly applicable to 18:1 *trans* fatty acids which fluctuate in human milk lipids according to their amount in the diet (18,28). Craig-Schmidt *et al.* (28) have reported a linear correlation between 18:1 *trans* in milk and in the diet among eight lactating women consuming different levels of partially hydrogenated fats. Using their linear equation, $Y = 1.49 + 0.42X$ [where Y and X represent the percentage 18:1 *trans* in total milk fat and dietary fat, respectively (28)], total *trans* intake can be estimated provided total *trans* fatty acids in diet and milk have similar relationship as 18:1 *trans*. Application of this equation indicates that HTM lactating women consumed diets in which 26.0% of fat was *trans* fatty acids. Thus, the total *trans* fatty acid intake for HTM women, ingesting a total energy of 2350 kcal in which 30% of the energy is derived from fat (29), could be estimated to be 7.7% of the total dietary energy or 20.3 g/person/d. Similarly, total *trans* fatty acid intake for MTM women could be estimated to be 3.9% total energy or 10.1 g/person/d, whereas total *trans* intake for LTM lactating women could be 1.1% total energy or 3.0 g/person/d. If the calculation is based on the mean *trans* fatty acid content (7.2%) for all the 198 human milk samples, then the mean total *trans* fatty acid intake for Canadian lactating women could be estimated to be 4.0% total energy or 10.6 g/person/d. This value is 16.5% higher than the value of 9.1 g/person/d estimated for the Canadian general population in 1981 (30). From data reported in four studies involving up to 57 breast milk samples (16,18,31,32), a mean value of 4.5 g/person/d can be deduced for lactating women in the United States. A similar *trans* fatty acid consumption has been estimated for British women (33). Thus, these data may suggest that Canadian women are consuming more *trans* fatty acids than are women in the United States or Britain. However, the estimated *trans* intake for women in the United States is far below the recent estimates of 12.5–15.2 g (34) or 8.1 g/person/d (35,36) for the general United States population.

In the present study a correlation between total *trans* fatty acids and total milk fat content was not found, which may indicate that dietary *trans* fatty acids do not suppress milk fat production in humans. This suggestion is consistent with the findings of Jensen *et al.* (37) for United States women. These findings with humans are also consistent with those in a laboratory study of ours in which partially hydrogenated canola oil did not suppress percent fat in lactating rats (Chen and Ratnayake, unpublished data). Similar observation was found in swine (23). Contrary to these findings, suppression of milk fat percentage by dietary *trans* fatty acids has been reported in mice (21,22). Thus, it appears that the influence of dietary

trans fatty acids on fat production in milk may be species-dependent.

The 18:1 *trans* isomer distribution was remarkably similar to that of partially hydrogenated vegetable oils. Since *trans* fatty acids are not *de novo* synthesized, the *trans* isomer distribution is a reflection of that in the diet. Under conditions of energy deficiency, lactating women produce milk which resembles the fatty acid pattern of their adipose tissue, but when energy is adequate, the dietary fatty acids are the major influence (38). The fatty acid composition of adipose tissue has also been suggested to reflect the long-term fatty acid profile of the diet (39–43). We have found a similar 18:1 *trans* isomer pattern between partially hydrogenated vegetable oil and adipose tissue in Canadian subjects (Chen, Ratnayake, Fortier, Ross, and Cunnane, unpublished data). A similar observation was also made by Ohlrogge *et al.* (20) for the United States population. Although complete dietary records were unavailable in the present study, this close correlation between the distribution of individual 18:1 isomers in partially hydrogenated vegetable oils and in human milk fat or adipose tissue, suggests that the major source of these fatty acids in the Canadian population is partially hydrogenated vegetable oil, whereas contribution from dairy products is relatively minor.

It is difficult to compare the pattern of 18:1 *cis* isomers in human milk with that in the diet because of the *de novo* synthesis of Δ9*c* and Δ11*c* isomers. However, comparison of the relative proportions of Δ8*c*, Δ9*c* and Δ12*c* isomers in partially hydrogenated vegetable oils with those in human milk indicates that Δ10*c* isomer is preferentially metabolized or discriminated by mammary gland. The low incorporation or apparent selective metabolism of the Δ10*c* isomer in human milk fat was also observed in human (20,44) and animal tissues (45,46).

Chappell *et al.* (19) studied the influence of margarine on fatty acid composition and found a significant elevation in milk 18:2n-6 levels with a depression in 18:1 *trans* following ingestion of margarine containing lower levels 18:1 *trans* fatty acids. This pattern in milk fatty acid was reversed by feeding margarine high in *trans* fatty acids and low in 18:2n-6 (19). We also found a similar inverse relationship between 18:2n-6 and 18:1 *trans* in human adipose tissue (Chen, Ratnayake, Fortier, Ross, and Cunnane, unpublished data). The linear inverse relationship between 18:2n-6 or 18:3n-3 and 18:1 *trans* isomers observed in this study indicates the elevation of *trans* fatty acids in Canadian human milk is at the expense of 18:2n-6, 18:3n-3 because high *trans* products contain less 18:2n-6 and 18:3n-3.

Many geometric and positional isomers of 18:2n-6 are formed during partial hydrogenation of vegetable oils (13,14) or during the deodorization of oils (47,48), 18:2Δ9*c*,13*t*, 18:2Δ9*c*,12*t* and 18:2Δ9*t*,12*c* are the major 18:2 isomers. Significant levels of these isomers are present in margarine and other dietary fats (13,14). The interest in these 18:2 isomers is related to their possible chain-elongation and desaturation to unusual 20:4 isomers (11,12,15). In essential fatty acid-deficient rats fed partially hydrogenated soybean oil, the formation of 20:4 isomers was extensive and was even more than the arachidonic acid produced (11). We have studied the relationship between chain-elongation and desaturation of the 18:2 isomers and dietary level of 18:2n-6 in rats. Our data have shown that elongation and desaturation of these isomers are inversely related to the level of 18:2n-6 in the diet (49). In another study (15), we found that 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t* were metabolized to 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t*, respectively, even under sufficient intake of 18:2n-6 (1.46% energy). Although these unusual 20:4 isomers were less than 0.4% of the total liver phospholipids, it is conceivable that they might exert some physiological effects. For example, a *trans* isomer of docosahexaenoic acid (DHA) derived from a mono *trans* isomer of 18:3n-3 has been found to incorporate into the retina of rats (50) and consequently altered the electroretinograph (51). We found that several milk samples in the present study had as much as 1.7% of total fat as 18:2 isomers. It is generally believed that dietary *trans*/*cis* isomers of 18:2n-6 and 18:3n-3 are not elongated and desaturated to C_{20} and C_{22} polyunsaturated fatty acids in humans, under conditions of adequate intake of essential fatty acids (5,6,32,52). Contrary to this, Chardigny *et al.* (53) found *trans* isomers of eicosapentaenoic acid and DHA in human platelets. Although 20:4 *trans* isomers are not present in human milk (present study), future studies should focus on the possible formation of C_{20} and C_{22} *trans* isomers in infants ingesting HTM. It will also be necessary to determine whether or not incorporation of such *trans* isomers into infants tissues may have physiological implications.

In addition to the possible chain-elongation and desaturation of 18:2 isomers, some 18:1 *cis* isomers may also be metabolized to unusual C_{20} isomers. For example, our previous study showed that 18:1Δ12*c* was desaturated/elongated subsequently to 20:2Δ8*c*,14*c* and 20:2Δ5*c*,8*c*,14*c* in rats fed the partially hydrogenated oils under sufficient intake of 18:2n-6 (15). In the human milks we analyzed, 18:1Δ12*c* accounted for 2.0% of total 18:1 *cis* fatty acids (Fig. 4). In addition, its two corresponding C_{20} metabolites were found to account for 0.2% of total milk fat (Table 1). The presence of the two metabolites in human milk suggests that the metabolic pathway of 18:1Δ12*c* may be similar in rats and humans.

In conclusion, it is clear that *trans* fatty acid isomers present in partially hydrogenated vegetable oils are transferred to human milk through maternal diets and, subsequently, to infants. Feeding diets high in *trans* fatty acids to newborn animals led to rapid accumulation of *trans* fatty acids in adipose tissue, liver and heart (54–56). The significance of an early supply of *trans* fatty acids in large amounts to human neonates is still unknown.

ACKNOWLEDGMENTS

Z.Y. Chen was a T.K. Murray Fellow of the National Institute of Nutrition. The authors thank Bureau of Field Operations and Dr. W.H. Newsome for procuring the samples. Thanks are also due to the

mothers who donated milk samples. T. Mag of CanAmera Food, Inc. (Toronto, Ontario, Canada) is thanked for supplying partially hydrogenated canola and soybean oil basestocks.

REFERENCES

1. Mensink, R.P., and Katan, M.B. (1990) *N. Engl. J. Med. 323,* 439–445.
2. Judd, J.T., Clevidence, B.A., Muesing, R.A., Wittes, J., Sunkin, M.E., and Podczasy, J.J. (1994) *Am. J. Clin. Nutr. 59,* 861–868.
3. Willet, W.C., Stampfer, M.J., Manson, J.E., Golditz, G.A., Speizer, F.E., Rosner, B.A., Sampson, L.A., and Hennekens, C.H. (1993). *Lancet 341,* 581–585.
4. Siguel, E.N., and Lerman, R.H. (1993) *Am. J. Cardiol. 71,* 916–920.
5. Emken, E.A. (1983) *J. Am. Oil. Chem. Soc. 60,* 995–1004
6. Senti, F., *Health Effects of Dietary* trans *Fatty Acids,* Bethesda, MD, Federation of American Societies for Experimental Biology, August 1985 (Report of HHS Contract No. FDA 223-87-2020).
7. Koletzko, B. (1992) *Acta. Paediatr. 81,* 302–306.
8. Koletzko, B., and Muller, J. (1990) *Biol. Neonate 57,* 172–178.
9. Anderson, R.L., Fullmer, C.S., and Hollenbach, E.J. (1975) *J. Nutr. 105,* 393–400.
10. Beyers, E.C., and Emken, E.A. (1991) *Biochim. Biophys. Acta. 1082,* 275–284.
11. Holman, R.T., Pusch, F., Svingen, B., and Dutton, H.J. (1991) *Proc. Natl. Acad. Sci. (USA) 88,* 4830–4834.
12. Hill, E.G., Johnson, S.B., Lawson, L.D., Mahfouz, M.M., and Holman, R.T. (1982) *Proc. Natl. Acad. Sci. (USA) 79,* 953–957.
13. Ratnayake, W.M.N., Hollywood, R., O'Grady, E., and Pelletier, G. (1993) *J. Am. Coll. Nutr. 12,* 651–660.
14. Ratnayake, W.M.N., and Pelletier, G. (1992) *J. Am. Oil. Chem. Soc. 69,* 95–105.
15. Ratnayake, W.M.N., Chen, Z.Y., Pelletier, G., and Weber, D. (1994) *Lipids 29,*707–714.
16. Picciano, M.F., and Perkins, E.G. (1977) *Lipids 12,* 407–408.
17. Koletzko, B., Maria, M., and Bremer, H.J. (1988) *Am. J. Clin. Nutr. 47,* 954–959.
18. Atichison, J.M., Kunkley, W.L., Canotly, N.L, and Smith, L.M. (1977) *Am. J. Clin. Nutr. 30,* 2006–2015.
19. Chappell, J.E., Clandinin, M.T., and Kearney-Volpe, K. (1985) *Am. J. Clin. Nutr. 42,* 49–56.
20. Ohlrogge, J.B., Emken, E.A, and Gulley, R.M. (1981) *J. Lipid Res. 22,* 955–960.
21. Teter, B.B., Sampugna, J., and Keeney, M. (1990) *J. Nutr. 120,* 818–824.
22. Teter, B.B., Sampugna, J., and Keeney, M. (1992) *Lipids 27,* 912–916.
23. Pettersen, J., and Opstvedt J. (1991) *Lipids 26,* 711–717.
24. Mes, J. (1981) *Internat. J. Environ. Anal. Chem. 9,* 283–299.
25. Chen, Z.Y., and Cunnane, S.C. (1992) *Am. J. Physiol. 27,* 912–916.
26. Ratnayake, W.M.N., and Beare-Rogers, J.L. (1990) *J. Chromatogr. Sci. 28,* 633–639.
27. Ackman, R.G., Sebedio, J.L., and Ratnayake, W.N. (1981) *Methods Enzymol. 72,* 253–276.
28. Craig-Schmidt, M.C., Weete, J.D., Faircloth, S.A, Wickwire, M.A, and Livant, E.J. (1984) *Am. J. Clin. Nutr. 39,* 778–786.
29. Health and Welfare Canada (1990) *Nutrition Recommendations: The Report of the Scientific Review Committee,* Canadian Government Publishing Centre, Ottawa, Canada.
30. Brisson, G.J. (1981) in *Lipids in Human Nutrition,* Jack K Burgess, Inc., pp. 41–71, Englewood.
31. Finley, D.A., Lonnerdal, B., Dewey, K.G., and Grivetti, L.E. (1985) *Am. J. Clin. Nutr. 41,* 787–800.
32. Hunderieser, K.E., Clark, R.M., and Brown, P.B. (1983) *J. Ped. Gastro. Nutr. 2,* 635–639.
33. Gregory, J., Foster, K., Tyler, H., and Wiseman, M. (1990) *The Dietary and Nutritional Survey of British Adults,* The Social Survey Division, Office of Population Censes and Survey, The United Kingdom.
34. Enig, M.G., Atal, S., Keeney, L., and Sampugna, J. (1990) *J. Am. Coll. Nutr. 9,* 471–486.
35. Hunter, J.E., and Applewhite, T.H. (1986) *Am. J. Clin. Nutr. 44,* 707–717.
36. Hunter, J.E., and Applewhite, T.H. (1991) *Am. J. Clin. Nutr. 54,* 363–369.
37. Jensen, R.G., Hagerty, M.M., and McMahon, K.E. (1978) *Am. J. Clin. Nutr. 31,* 990–1016.
38. Insull, W., Hirsh, T.J., James, T., and Ahrens, E.H. (1959) *J. Clin. Invest. 28,* 443–450.
39. Martin, F.C., Bougnoux, P., Fignon, A., Theret, V., Antoine, J.M., Lamisse, F., and Gouet, C. (1993) *Am. J. Clin. Nutr. 58,* 653–659.
40. Dayton, S., Hashimoto, S., Dixon, W., and Pearce, M.L. (1966) *J. Lipid Res. 7,*103–106.
41. Beynen, A.C., Herms, R.J.J., and Hautvost, J.G.A.J. (1980) *Am. J. Clin. Nutr. 33,* 81–85.
42. Hirsh, J.J., Farquhar, W., Ahrens, E.H., Peterson, M.L., and Stoffel, W. (1956) *Am. J. Clin. Nutr. 8,* 499–511.
43. Hudgins, L.C., Hirsch, J., and Emken, E.A. (1991) *Am. J. Clin. Nutr. 53,* 474–482.
44. Emken, E.A., Rohwedder, W.K., Adlof, R.O., Dejarlis, W.J., and Gully, R.M. (1985) *Biochem. Biophys. Acta. 836,* 233–245.
45. Wood, R. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J, eds.) pp. 213–276, American Oil Chemists' Society, Champaign.
46. Reichwald-Hacker, I., Grosse-Oetringhaus, S., Kiewitt, I., and Mukherjee, K.D. (1979) *Biochim. Biophys. Acta. 575,* 327–334.
47. Ackman, R.G., Hooper, S.N., and Hooper, D.L. (1974) *J. Am. Oil. Chem. Soc. 51,* 42–49.
48. Wolf, R.L. (1992) *J. Am. Oil. Chem. Soc. 69,* 106–110.
49. Chen, Z.Y., Pelletier, G., Hollywood, R., and Ratnayake, W.M.N. (1994) *INFORM 5,* 473.
50. Grandgirard, A., Bourre, J.M., Julliard, F., Homayoun, P., Dumont, O., Piciotti, M., and Sebedio, J.L. (1994) *Lipids 29,* 251–258.
51. Chardigny, J.M., Bonhomme, B., Sebedio, J.L., Juaneda, P., Doly, M., and Grandgirard, A. (1994) *INFORM 5,* 472.
52. Applewhite, T.H., and Hunter, J.E. (1992) *Lipids 27,* 486.
53. Chardigny, J.M., Sebedio, J.L., Juaneda, P., Vatele, J.M., and Grandgirard, A. (1993) *Nutr. Res. 13,* 1105–1111.
54. Pettersen, J., and Opstvedt, J. (1989) *Lipids 24,* 616–624.
55. Moore, C.E., and Dhopeshwarkar, G.A. (1980) *Lipids 15,* 1023–1028.
56. Rastogi, B.K., Fryer, B.A., Allee, F., and Fryer, H.C. (1980) *Nutr. Rep. Inter. 22,* 925–933.

[Received August 26, 1994, and in revised form November 15, 1994; Revision accepted November 23, 1994]

Thermal Acclimation and Dietary Lipids Alter the Composition, But Not Fluidity, of Trout Sperm Plasma Membrane

C. Labbe[a,*], G. Maisse[a], K. Müller[b,1], A. Zachowski[b], S. Kaushik[c] and M. Loir[a]

[a]Laboratoire de Physiologie des Poissons, INRA, Rennes, France,

[b]Institut de Biologie Physico-Chimique, Paris, France, and [c]Laboratoire de Nutrition des Poissons, INRA, Saint Pée sur Nivelle, France

ABSTRACT: The effect of a long-term adaptation of rainbow trout to 8 and 18°C combined with a corn oil- or a fish oil-supplemented diet on the characteristics of the spermatozoan plasma membrane was investigated. The experiment lasted up to 22 mon during which spermatozoa were collected from the mature males. Spermatozoan plasma membranes were isolated by nitrogen cavitation, and the cholesterol content, phospholipid composition and fatty acid pattern were investigated. Membrane viscosity was assessed on whole cells by electron spin resonance using spin-labeled phospholipids. Neither diet nor rearing temperature influenced the cholesterol content of the plasma membrane nor the phospholipid class distribution. The rearing temperature of the broodstock only slightly affected the phospholipid fatty acids. A minor decrease in 18:0 and increase in monounsaturated fatty acids was observed for the cold-adapted fish. These modifications were not sufficient to affect membrane fluidity, and we conclude that trout spermatozoa do not display any homeoviscous adaptations in these conditions. On the contrary, the dietary fatty acid intake greatly modified the fatty acid profile of plasma membrane phospholipids. The fish oil-fed trout displayed a much higher n-3/n-6 fatty acid ratio than did the corn oil-fed ones, but the 22:6n-3 levels remained unchanged. Modifications in plasma membrane composition by the diet were obtained although neither of the two diets was deficient in essential fatty acids. The enrichment in n-3 fatty acids, however, did not affect plasma membrane fluidity which was unchanged by the diets.
Lipids 30, 23–33 (1995).

The quality of the spermatozoan plasma membrane in rainbow trout is a key factor in protecting the cell from the extracellular environment, be it the hypo-osmotic water into which milt is ejaculated or the media and extenders used in artificial fertilization. The importance of trout sperm plasma membrane integrity in resistance to stress such as osmotic shock or cold shock was confirmed by Maléjac *et al.* (1). Lipids, particularly phospholipids and cholesterol, are important structural elements of the cell membrane. It has been demonstrated in many vertebrate species that the resistance of sperm to cold shock depends on their cholesterol content (2–4), the cellular level of unsaturated fatty acids (5), and the phase transition temperature of spermatozoan glycolipids (6). In artificial membrane vesicles, the fatty acid composition has been shown to affect bilayer permeability and the ability of the membrane to fuse (7,8). Fatty acid composition also influences the osmotic fragility of erythrocytes, which additionally depends on the nature of phospholipid polar head groups (9,10). Several reviews have extensively covered the relationships between membrane composition (i.e., fatty acid unsaturation and acyl chain length, cholesterol, phospholipids, and proteins) and the physical parameter often referred to as "membrane fluidity" (11–13).

In mammals, changes in plasma membrane composition through dietary manipulation are well documented (reviewed in Ref. 11). In fish, many studies have shown that the fatty acid composition of the diet influences tissue lipids, particularly the fatty acid pattern in phospholipids (for review, see Refs. 14,15). The lipid composition of the broodstock diet has been reported to affect the fatty acid profile of trout eggs and spermatozoa (16,17) and of gilthead sea bream eggs (18). These studies, however, compared essential fatty acid (EFA)-deficient and non EFA-deficient diets and concerned whole cells. To our knowledge, the only data available in fish membranes concern the brush border membrane of trout and carp intestine (19–21).

The temperature at which fish are reared also affects the lipid composition of the cells. Cold acclimation of these poikilothermic animals has been shown to increase the proportions of unsaturated fatty acids and phosphatidylethanolamine and to decrease the proportions of saturated fatty acids, phosphatidylcholine, sphingomyelin, and cholesterol (13). These

*To whom correspondence should be addressed at the Laboratoire de Physiologie des Poissons, INRA, Campus de Beaulieu, 35042 Rennes Cedex, France.

[1]Present address: Institut für Fortpflanzung Landwirtschaftlicher Nutztiere, Schönow e.V., 16321 Schönow, Germany.

Abbreviations: BCA, bicinchoninic acid; BHT, butylated hydroxytoluene; DFP, diisopropyl fluorophosphate; EFA, essential fatty acid; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; MUFA, monounsaturated fatty acid; SE, standard error; SL-PC, spin-labeled phosphatidylcholine; SL-PS, spin-labeled phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

Copyright © 1995 by AOCS Press

modifications were assumed to increase membrane fluidity and to maintain normal cellular functions at low environmental temperature.

It is not known how the rearing temperature and changes in the dietary fatty acid profiles affect the lipids in the membranes of rainbow trout spermatozoa. The present study was undertaken to investigate the combined effects of temperature and dietary lipids on the cholesterol content, phospholipids, and fatty acid composition of phospholipid classes in the plasma membranes of rainbow trout spermatozoa. To this end, broodstock were reared at two water temperatures (8 and 18°C), and the diets were enriched with either linoleic acid or n-3 polyunsaturated fatty acids (PUFAs). For each set of rearing conditions, the biophysical state of the spermatozoan membrane was assessed by electron spin resonance (ESR) to estimate membrane fluidity.

MATERIALS AND METHODS

Animals, rearing temperatures and diets. One-year-old juvenile rainbow trout (*Oncorhynchus mykiss*) specimens of a winter strain, weighing about 100 g and reared at the INRA experimental farm at Donzacq, France (18°C water), were divided into four groups. Two groups were transferred to the INRA experimental farm at Lees-Athas, France (8°C water). In each farm, the trout were fed one of two experimental isoenergetic diets, in the form of pellets. Diet compositions are shown in Table 1. The relative percentages of n-3 fatty acids (0.5% in the corn oil diet and 1.1% in the cod liver oil diet) and n-6 fatty acids (3.7 and 1.36% respectively) met the known EFA requirements of broodstock fish. The corn oil diet was enriched in linoleic acid (three times more than in the cod liver oil diet), the cod liver oil diet in n-3 fatty acids (2.3-fold with respect to the former). The ratio of highly PUFA (at least four unsaturations) to saturated fatty acids was 2-fold lower in the corn oil diet (0.21) than in the cod liver oil diet (0.40). To obtain the same growth rate at both rearing temperatures, the fish reared at 18°C were rationed.

The first sexual maturity of the fish occurred 9 mon after the beginning of the experiment. Their mean weight was 1 kg and they were then two years old. Sperm was collected by hand stripping. For each rearing condition, sperm from six to nine fish were pooled. Each fish contributed the same sperm volume in the pool. This year, we obtained one to two pools of sperm, depending on the group. These pools were used for the determination of the lipid composition of plasma membrane. After another year under these rearing conditions (total of 22 mon), fish reached their second sexual maturity as they became three years old. Sperm was collected again and pooled in the same conditions. The two pools obtained this year were used for both the lipid composition and the fluidity studies on the plasma membrane. Since no difference in the lipid composition appeared between the two breeding seasons, the corresponding data were not separated in Tables 3 to 7.

Membrane preparation. Pure spermatozoan plasma membranes were prepared as previously described (22). Briefly, washed spermatozoa were subjected to a 900 psi nitrogen pressure for 20 min. Sperm extrusion lasted one minute, and a crude membrane preparation was collected after centrifuging (500 × *g* for 20 min) on a 1M sucrose cushion. Plasma membranes were separated from contaminating nuclear membranes, mitochondria, and flagella by sucrose density ultracentrifugation (54,000 × *g* for 2 h) on a subcontinuous gradient (1.8 to 0.25 M). The purity of the plasma membrane fraction was assessed by electron microscopy and by enzyme marker assays (22). The plasma membrane fraction showed a 4- to 5-fold enrichment in 5′-nucleotidase (EC 3.1.3.5; plasma membrane marker) activity, and no succinic dehydrogenase (EC 1.3.99.1; mitochondrial marker) activity was detected.

TABLE 1
Composition of Experimental Diets

Ingredients (wt%)[a]	Corn oil diet	Cod liver oil diet
Fish meat	45	45
Wheat starch (native)	20	20
Soybean LTI	18	18
Pre-gelatinized starch	5	5
Mineral mix (INRA[b], France)	2	2
Vitamin mix (INRA[b], France)	2	2
Sodium alginate	2	2
Corn oil	6	0
Cod liver oil	0	6
Antioxidant (ethoxyquin, ppm)	100	100
Protein (%)	43	43
Lipid (%)	11	11
Digestible energy (kJ/g)	15.5	15.5
Major fatty acids (molar % of the total)		
14:0	2.0	6.0
16:0	17.6	24.6
16:1	2.2	8.1
18:0	2.9	4.0
18:1	23.8	22.3
18:2n-6	44.8	15.2
18:3n-3	1.7	2.2
18:4n-3	0.3	1.3
20:1	0.2	2.3
20:4n-6	0.2	0.4
20:4n-3	—	0.3
20:5n-3	2.0	5.5
22:1	—	1.6
22:5n-3	0.2	0.5
22:6n-3	2.0	5.8
Total n-3	6.3	15.5
Total n-6	45.0	15.6
n-3/n-6	0.1	1.0
UI	139.3	128.6

[a]LTI, Low Trypsin Inhibiteur extruded full fatty soybean; UI, unsaturation index = relative percentage of each unsaturated fatty acid times the number of double bonds for that particular fatty acid.
[b]Mineral mix contained the following ingredients (g/kg mix):calcium carbonate, 215; magnesium hydroxide, 124; KCl, 90; ferric citrate, 20; KI, 0.4; NaCl, 40; calcium hydrogen phosphate ($CaHPO_4$), 500; copper sulfate, 3; zinc sulfate, 4; cobalt sulfate, 0.2; manganese sulfate, 3.
[c]Vitamin mix contained the following diluted in cellulose (g/kg mix):vitamin A (500,000 IU/g), 1.5; vitamin D_3 (100,000 IU/g), 1.5; vitamin E (500 IU/g), 6; vitamin K, 0.25; thiamine, 0.75; riboflavin, 1.5; pyridoxine, 0.75; nicotinic acid, 8.75; vitamin C, 25; folic acid, 0.25; vitamine B_{12} (1000 mg/kg), 2.5; inositol, 50; biotin (2%), 6.25; calcium pantothenate, 2.5; choline (50%), 200.

Lipid extraction and analysis. One volume of plasma membrane suspension was vigorously mixed with four volumes of chloroform/methanol (2:1, vol/vol) (23) containing 0.02% butylated hydroxytoluene (BHT) as an antioxidant. The lower phase was carefully separated, dried, and dissolved in chloroform with 0.02% BHT. Total cholesterol was determined enzymatically (Biochemica test combination; Boehringer, Mannheim, Germany) in 96-well plates after lipid extraction. Phospholipids were quantitated by colorimetric determination of phosphorus according to Bartlett (24) after hydrolysis with sulfuric acid. Phospholipids were separated from neutral lipids and glycolipids according to Linard (25). After adsorption of the sample to the Sep-Pak silica columns (Waters Associates, Millipore Corporation, Milford, MA), 20 mL chloroform were pushed through the cartridge, followed by 10 mL acetone and 20 mL methanol. Phospholipid classes were separated by high-performance liquid chromatography (HPLC) on a Zorbax silica column (250 mm length, 4.6 mm internal diameter, 5 μm particle size, Societe Francaise de Chromatographie, Eragny, France) on line with a LCD/Milton Roy 3000 detector (Riviera Beach, CA) at 205 nm. The mobile phases were: Solvent A, *n*-hexane/2-propanol (3:2, vol/vol), and Solvent B, *n*-hexane/2-propanol/water (3:2:0.275, by vol) at a flow rate of 1.5 mL/min. After injection of phospholipids, the column was eluted for 9 min with 50% A:50% B. The proportion of B was increased in 5 min to 78%, maintained for 9 min, increased to 100% in 2 min, and maintained for another 14 min. Finally, the proportion of B was decreased to 50% in 4 min, maintained for 9 min, and elution was stopped. Under these conditions, the various classes of phospholipids eluted within 50 min. Quantitation of these classes was performed by phosphorus assay.

Fatty acid distribution in the phospholipid classes was determined by gas–liquid chromatography of the fatty acid methyl esters obtained after transmethylation (26). For each class, 1 mg phospholipid in 1 mL methanol and 8 mL 12 N hydrochloric acid chloroform/2,2-dimethoxypropane (9.87:86.25:3.95, by vol) with 0.02% BHT were mixed in screw-cap vials which were then closed under a flush of nitrogen and heated at 65°C. The reaction was carried out for 3 h for total phospholipids, 4 h for phosphatidylethanolamine and phosphatidylcholine, 4.5 h for phosphatidylinositol, and 5 h for phosphatidylserine. After the tubes had cooled, fatty acid methyl esters were extracted in 4 mL *n*-hexane, dried and dissolved in isooctane.

Á Chrompack CP 9000 gas chromatograph (Les Ullis, France) equipped with a splitter injector, a flame-ionization detector, and a CP wax 52 CB bonded fused silica capillary column (50 m ¥ 0.25 mm internal diameter) was used for fatty acid methyl ester analysis. The carrier gas was hydrogen, at a flow rate of 1.5 mL/min. The injector and detector were set at 250 and 270°C, respectively, and the oven temperature was increased from 180 to 225°C at a rate of 4°C/min. Peaks were identified by comparing their retention times with those of authentic fatty acid methyl esters, and areas were calculated on a Spectra-Physics integrator (SP4400, Chrompack, Les Ullis, France).

Spermatozoan spin-labeling. The spin-labeled analogues of phospholipids used in these experiments are 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine (SL-PC) and -phosphatidylserine (SL-PS) (see Ref. 27 for their synthesis description). The required amount of spin-labeled analog (corresponding to 1.6% of total membrane phospholipids) from a chloroform solution was deposited in a tube and dried under nitrogen. The dried film was resuspended in one volume of seminal-fluid-like mineral medium (28) consisting of 110 mM NaCl, 28.3 mM KCl, 1.1 mM $MgSO_4$, 1.8 mM $CaCl_2$ and 20 mM tris buffer (pH 9). One volume of spermatozoa in suspension ($2 \cdot 10^{10}$ cells/mL) and one volume of diisopropyl fluorophosphate (DFP) 5 mM were mixed with the spin-labeled phospholipid. Cell suspension was incubated for 30 min at room temperature to allow SL-PS to equilibrate in the membrane inner leaflet. ESR spectra were recorded at 4, 8, 13, 18 and 24°C after re-oxidation of nitroxides by 12 mM potassium ferricyanide. All ESR measurements were performed with a Varian E-109 spectrometer (Palo Alto, CA) equipped with a temperature control device. To compare the mobility of the spin probe in the different conditions, the apparent rotational correlation time was estimated as described (29).

Protein content. Membrane suspensions were digested in 1 M sodium hydroxide overnight at room temperature, and the protein content was determined according to Smith *et al.* (30) using the Pierce bicinchoninic acid (BCA) protein assay reagent (Rockford, IL).

Statistical analysis. Sperm from six to nine fishes were pooled for each individual analysis. Unless otherwise mentioned, the results shown are the means of three to four of these analyses ± standard error (SE). The statistical significance of differences between different groups was determined by the Mann-Whitney test (rank-sum test) (31). This test was performed by comparing the two diets, irrespective of the rearing temperature, and the two temperatures, irrespective of the diet in order to increase the sample number (from 3–4 to 6–8) and to allow this kind of analysis. The total number of fish involved in each group varied from 17 to 34.

RESULTS

Neither the lipid content of the two diets nor the rearing temperature had any significant effect on the phospholipid or cholesterol contents (relative to protein) of the spermatozoan plasma membrane (Table 2). The high SEs are due to high individual variations and made it difficult to assess the statistical significance of small differences between groups.

The different broodstock rearing conditions did not result in significantly different phospholipid class distributions in spermatozoan plasma membranes (Table 3). Whatever the diet or rearing temperature, phosphatidylcholine was the major phospholipid (46.5 to 54%), followed by phosphatidylethanolamine (29.5 to 33%) and phosphatidylserine (9.5 to 10.3%). Sphingomyelin was present in minor proportions (1.0 to 2.7%), and diphosphatidylglycerol was hardly detected (1.0%).

TABLE 2
Lipid Composition of Spermatozoan Plasma Membrane from Trout Reared at 8 or 18°C and Fed with Either a Corn Oil-Supplemented or a Cod Liver Oil-Supplemented Diet Before Spawning[a]

	8°C Reared		18°C Reared	
	Corn oil diet	Cod liver oil diet	Corn oil diet	Cod liver oil diet
Phospholipid (μmol/mg protein)	3.30 ± 0.59	2.81 ± 0.48	2.66 ± 0.31	2.93 ± 0.36
Cholesterol (μmol/mg protein)	1.06 ± 0.21	0.99 ± 0.28	0.92 ± 0.12	0.74 ± 0.09
Cholesterol/phospholipid (molar ratio)	0.33 ± 0.08	0.35 ± 0.06	0.35 ± 0.04	0.26 ± 0.06

[a]Values are means of three to four pools ± SE of six to nine fish sperm. No statistical significance was observed between the groups (Mann-Whitney test).

TABLE 3
Phospholipid Class Distribution (% of total phospholipids) of Spermatozoan Plasma Membrane from Trout Reared at 8 or 18°C and Fed with Either a Corn Oil-Supplemented or a Cod Liver Oil-Supplemented Diet Before Spawning[a]

	8°C Reared		18°C Reared	
	Corn oil diet	Cod liver oil diet	Corn oil diet	Cod liver oil diet
Phosphatidylcholine	48.67 ± 2.52	51.24 ± 4.15	46.51 ± 3.89	54.00 ± 5.66
Phosphatidylethanolamine	31.33 ± 3.06	31.43 ± 3.18	32.99 ± 1.90	29.50 ± 3.54
Phosphatidylserine	10.33 ± 1.53	9.89 ± 0.65	10.01 ± 2.48	9.50 ± 2.12
Phosphatidylinositol	3.33 ± 1.53	2.73 ± 0.53	4.50 ± 1.27	2.50 ± 0.71
Lysophosphatidylcholine	3.00 ± 0.00	2.97 ± 1.15	3.51 ± 1.03	2.50 ± 0.71
Sphingomyelin	2.67 ± 1.53	0.99 ± 0.02	1.00 ± 0.82	1.00 ± 0.00
Diphosphatidylglycerol	0.67 ± 0.58	0.74 ± 0.50	1.50 ± 1.29	1.00 ± 0.00

[a]Values are means of three to four pools ± SE of six to nine fish sperm. No statistical significance was observed between the groups (Mann-Whitney test).

We investigated the fatty acid profiles of the four main phospholipid classes: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol (Tables 4 to 7). Palmitic acid was present in high amounts in trout spermatozoan plasma membrane, accounting for 40% of the total fatty acids in phosphatidylcholine (Table 4), 20% in phosphatidylethanolamine (Table 5), and over 10% in phosphatidylserine (Table 6) and phosphatidylinositol (Table 7). Oleic acid was also a major fatty acid in all four classes. In phosphatidylserine, 25% of the total fatty acids was 18:1n-9, and in the remaining classes the percentage was 12 to 20%. Phosphatidylserine was characterized by high proportions of 22:6n-3 (25%), and this fatty acid accounted for 10 to 15% of the total fatty acids in phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine. Of the four classes, phosphatidylcholine, the major phospholipid, had the lowest unsaturated fatty acid (UFA) to saturated fatty acids (SFA) ratio and the lowest unsaturation index. The high UFA to SFA ratio in phosphatidylserine is due to the fact that half of the phosphatidylserine fatty acids were 18:1n-9 and 22:6n-3 (Table 6).

The four phospholipid classes all exhibited the same general pattern of fatty acid change in response to reduced water temperature and to dietary modifications. In phosphatidylcholine, the proportion of 18:0 was lower at a rearing temperature of 8°C while the proportion of monounsaturated fatty acids (MUFA) was higher (Table 4). This slightly but significantly increased the MUFA to PUFA ratio but did not influence the UFA to SFA ratio or the unsaturation index. The effect of diet on phosphatidylcholine was as follows: the high amounts of 18:2n-6 in corn oil increased not only the plasma membrane 18:2n-6 levels (2-fold) but also the proportions of all n-6 PUFA (20:2n-6, 20:4n-6, and 22:5n-6 2-fold), the greatest difference between the two dietary groups being the 4-fold enrichment in 20:3n-6 in corn oil-fed trout (Table 4). The high proportions of 18:2n-6 and 20:4n-6 in trout fed corn oil as compared to trout fed cod liver oil resulted in a lower MUFA to PUFA ratio in the former. The corn oil diet had only one-third as much 14:0 and one-fourth as much 16:1 as did the cod liver oil diet (Table 1), but the plasma membrane phosphatidylcholines in corn oil-fed trout contained two-thirds as much 14:0 as found in that from fish oil-fed trout. The high proportion of n-3 PUFAs in the cod liver oil diet (two times more 20:5n-3 and 22:5n-3 than in the corn oil diet) resulted in a similar enrichment of spermatozoan membrane phosphatidylcholine in these fatty acids. Surprisingly, the 22:6n-3 levels were the same in both groups, although the cod liver oil-coated pellets were 3-fold richer in this fatty acid. Thus, phosphatidylcholine is the phospholipid class for which the difference in the n-3/n-6 ratio between the two diets is the highest and for which the UFA/SFA ratio is most affected by the diets. The resulting unsaturation index was slightly higher in the cod liver oil-fed fish, mainly due to the proportion of 20:5n-3, but this difference was observed mainly in fish reared at 18°C and was not significant.

Phosphatidylethanolamine was more unsaturated than phosphatidylcholine, the UFA/SFA ratio being about two times higher in phosphatidylethanolamine. The effects of di-

TABLE 4

Fatty Acid Composition of Phosphatidylcholines in Spermatozoan Plasma Membrane from Trout Reared at 8 or 18°C and Fed with Either a Corn Oil-Supplemented or a Cod Liver Oil-Supplemented Diet Before Spawning[a]

Fatty acid[b]	8°C Reared		18°C Reared	
	Corn oil diet	Cod liver oil diet	Corn oil diet	Cod liver oil diet
14:0	1.65 ± 0.35[c]	2.47 ± 0.09[d]	1.30 ± 0.12[c]	2.09 ± 0.48[d]
15:0	0.51 ± 0.09[c]	0.69 ± 0.03[d]	0.39 ± 0.05[c]	0.54 ± 0.11[d]
16:0	38.31 ± 2.68	38.89 ± 0.81	37.94 ± 1.65	38.57 ± 3.62
17:0	0.23 ± 0.02[c]	0.26 ± 0.01[d]	0.19 ± 0.01[c]	0.27 ± 0.02[d]
18:0	2.50 ± 0.13[a]	2.06 ± 0.13[a]	3.52 ± 0.43[b]	3.57 ± 0.22[b]
16:1n-9	1.21 ± 0.25[a,c]	1.13 ± 0.15[a,d]	1.00 ± 0.13[b,c]	0.40 ± 0.45[b,d]
16:1n-7	1.37 ± 0.15[a,c]	2.04 ± 0.16[a,d]	0.78 ± 0.11[b,c]	1.75 ± 1.00[b,d]
18:1n-9	13.20 ± 1.52	12.12 ± 0.43	12.34 ± 0.95	11.25 ± 0.74
18:1n-7	7.14 ± 0.92[a]	8.85 ± 1.49[a]	5.02 ± 0.86[b]	7.78 ± 1.55[b]
20:1n-9/7	0.29 ± 0.03	0.41 ± 0.09	0.38 ± 0.24	0.34 ± 0.02
18:2n-6	6.51 ± 0.45[c]	3.22 ± 0.33[d]	10.13 ± 0.49[c]	3.63 ± 0.27[d]
18:3n-3	0.20 ± 0.08	0.19 ± 0.07	0.24 ± 0.12	0.26 ± 0.02
20:2n-6	1.46 ± 0.28[c]	0.68 ± 0.16[d]	1.76 ± 0.28[c]	0.63 ± 0.01[d]
20:3n-6	1.20 ± 0.20[c]	0.32 ± 0.08[d]	2.08 ± 0.17[c]	0.42 ± 0.05[d]
20:4n-6	4.20 ± 0.77[c]	2.44 ± 0.18[d]	5.04 ± 0.75[c]	2.99 ± 0.52[d]
20:5n-3	6.16 ± 1.38[c]	10.41 ± 1.64[d]	4.45 ± 0.36[c]	10.84 ± 2.15[d]
22:3n-9	0.35 ± 0.18	0.33 ± 0.06	0.29 ± 0.09	0.41 ± 0.01
22:5n-6	0.43 ± 0.11[c]	0.24 ± 0.02[d]	0.57 ± 0.10[c]	0.27 ± 0.04[d]
22:5n-3	0.28 ± 0.07[c]	0.40 ± 0.03[d]	0.23 ± 0.04[c]	0.48 ± 0.14[d]
22:6n-3	11.62 ± 2.61	11.85 ± 0.48	10.87 ± 1.52	12.77 ± 3.55
Sum SFA	43.29 ± 3.01	44.41 ± 0.85	43.46 ± 1.44	45.09 ± 3.95
Sum MUFA	23.94 ± 2.61[a]	25.27 ± 1.79[a]	20.31 ± 1.56[b]	21.91 ± 2.76[b]
Sum PUFA	32.77 ± 5.24[c]	30.31 ± 2.15[d]	36.23 ± 2.98[c]	32.99 ± 6.71[d]
Sum n-3	18.37 ± 3.93[c]	23.00 ± 2.06[d]	15.89 ± 1.84[c]	24.50 ± 5.86[d]
Sum n-6	13.97 ± 1.44[c]	6.97 ± 0.58[d]	19.88 ± 1.25[c]	8.04 ± 0.85[d]
n-3/n-6	1.30 ± 0.14[c]	3.32 ± 0.38[d]	0.80 ± 0.06[c]	3.02 ± 0.41[d]
MUFA/PUFA	0.75 ± 0.17[a,c]	0.84 ± 0.12[a,d]	0.57 ± 0.09[b,c]	0.69 ± 0.22[b,d]
UFA/SFA	1.32 ± 0.17[c]	1.25 ± 0.04[d]	1.30 ± 0.08[c]	1.23 ± 0.19[d]
UI	167.17 ± 24.38	172.53 ± 9.28	165.39 ± 13.20	181.23 ± 32.96

[a,b]Significant effect ($P < 0.05$) of the rearing temperature between a and b inside one diet (Mann-Whitney test); [c,d]significant effect ($P < 0.05$) of the diet between c and d inside one rearing temperature (Mann-Whitney test).

[a]Values are means of three to four pools ± SE (molar %) of six to nine fish sperm.

[b]SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; UI, unsaturation index; PUFA, polyunsaturated fatty acid; UFA, unsaturated fatty acid.

etary n-3 and n-6 enrichment were about the same as for phosphatidylcholine. The proportion of 22:6n-3 was not affected. In phosphatidylethanolamine, however, the high proportion of 20:5n-3 in cod liver oil-fed trout resulted in an increased unsaturation index despite the high levels of 20:4n-6 and 18:2n-6 in corn oil-fed groups. Rearing at 18°C resulted in higher 18:0 and lower 16:0 levels than observed in 8°C reared trout, leading to a lower MUFA/PUFA ratio in the latter.

Phosphatidylserine is the phospholipid class in which fatty acids, excluding 18:0, were the most strongly influenced by the rearing temperature (Table 6). Amounts of all SFA possessing 14 to 17 carbons were higher in 18°C acclimated trout, resulting in a lower UFA/SFA ratio in this group. Palmitoleic acid levels were higher in the 18°C reared groups. The two diets affected the fatty acid profile of phosphatidylserine as described for phosphatidylcholine, with 22:6n-3 unchanged whatever the rearing conditions. Contrary to phosphatidylcholine, phosphatidylserine exhibited a lower MUFA/PUFA ratio in the cod liver oil-fed groups. The unsaturation index was unaffected by the rearing conditions.

In phosphatidylinositol, the five fatty acids, 16:0, 18:1n-9, 20:4n-6, 20:5n-3, and 22:6n-3, were present in proportions approaching 10% or more. The high levels of 16:0 and 18:0 made this phospholipid class very saturated, a fact reflected in the low unsaturation index. The diet mainly affected 20:4n-6 and 20:5n-3 levels, the 22:6n-3 level remaining unchanged. As observed for phosphatidylcholine and phosphatidylethanolamine, 18:0 levels were higher in the fish reared at 18°C, leading to a lower UFA/SFA ratio. As in phosphatidyl-serine and phosphatidylcholine, the unsaturation index was not affected by the rearing conditions.

The fact that 20:3n-9 was not detectable in all four phospholipid classes confirms that neither of the diets was EFA-deficient (32).

Membrane viscosity. Membrane viscosity was measured on sperm from each of the four experimental groups. ESR spectra were recorded with SL-PC and SL-PS at five different temperatures, namely at 4°C, the temperature at which spermatozoa are stored after collection; 8 and 18°C, the two rearing temperatures before spawning; 13°C, the temperature at which trout are usually reared in our laboratory; and at 24°C, the temperature used for thawing and fertilization after cryopreservation. The SL-PC reflected the microviscosity in the outer monolayer, and the SL-PS reflected that of the inner

TABLE 5
Fatty Acid Composition of Phosphatidylethanolamines in Spermatozoan Plasma Membrane from Trout Reared at 8 or 18°C and Fed with Either a Corn Oil-Supplemented or a Cod Liver Oil-Supplemented Diet Before Spawning[a]

Fatty acid[b]	8°C Reared		18°C Reared	
	Corn oil diet	Cod liver oil diet	Corn oil diet	Cod liver oil diet
14:0	1.32 ± 0.16	0.93 ± 0.37	0.79 ± 0.39	0.78 ± 0.01
15:0	0.67 ± 0.19	0.68 ± 0.34	0.49 ± 0.27	0.39 ± 0.03
16:0	21.50 ± 2.17[a]	20.22 ± 0.58[a]	19.10 ± 2.95[b]	17.87 ± 1.66[b]
17:0	0.32 ± 0.02[c]	0.38 ± 0.08[d]	0.28 ± 0.06[c]	0.31 ± 0.02[d]
18:0	3.48 ± 0.75[a]	2.94 ± 0.24[a]	4.69 ± 0.17[b]	4.88 ± 0.08[b]
16:1n-9	2.04 ± 0.64	1.50 ± 0.68	1.48 ± 0.92	1.00 ± 0.12
16:1n-7	0.48 ± 0.07[c]	0.86 ± 0.05[d]	0.30 ± 0.03[c]	0.62 ± 0.01[d]
18:1n-9	18.45 ± 3.62	17.88 ± 0.85	12.90 ± 1.96	18.55 ± 1.82
18:1n-7	1.51 ± 0.18[c]	2.24 ± 0.12[d]	1.28 ± 0.02[c]	2.38 ± 0.03[a]
20:1n-9/7	0.32 ± 0.05[c]	0.39 ± 0.05[d]	0.25 ± 0.05[c]	0.51 ± 0.15[d]
18:2n-6	9.68 ± 0.68[c]	6.68 ± 0.13[d]	13.04 ± 0.87[c]	7.32 ± 0.63[d]
18:3n-3	0.29 ± 0.04[c]	0.46 ± 0.11[d]	0.35 ± 0.10[c]	0.47 ± 0.05[d]
20:2n-6	0.77 ± 0.16[c]	0.46 ± 0.05[d]	1.16 ± 0.16[c]	0.50 ± 0.00[d]
20:3n-6	1.45 ± 0.51[c]	0.40 ± 0.14[d]	2.40 ± 0.35[c]	0.42 ± 0.01[d]
20:4n-6	8.84 ± 1.23[c]	5.17 ± 0.36[d]	12.88 ± 0.50[c]	6.13 ± 0.11[d]
20:5n-3	10.65 ± 2.04[c]	18.70 ± 0.92[d]	9.51 ± 0.85[c]	17.43 ± 0.65[d]
22:3n-9	0.28 ± 0.15	0.21 ± 0.08	0.17 ± 0.06	0.16 ± 0.02
22:5n-6	0.46 ± 0.06[c]	0.26 ± 0.04[d]	0.71 ± 0.14[c]	0.29 ± 0.06[d]
22:5n-3	0.24 ± 0.02[c]	0.43 ± 0.02[d]	0.28 ± 0.03[c]	0.52 ± 0.09[d]
22:6n-3	15.02 ± 1.68	16.85 ± 0.83	15.85 ± 1.80	17.55 ± 2.75
Sum SFA	27.65 ± 2.32	25.49 ± 1.48	25.87 ± 3.17	24.83 ± 1.60
Sum MUFA	24.21 ± 3.18[a]	24.25 ± 1.25[a]	17.18 ± 2.28[b]	23.78 ± 1.55b
Sum PUFA	48.14 ± 4.51	50.26 ± 1.79	56.95 ± 3.44	51.40 ± 3.15
Sum n-3	26.31 ± 3.07[c]	36.73 ± 1.53[d]	26.09 ± 2.07[c]	36.28 ± 3.56[d]
Sum n-6	21.33 ± 1.90[c]	13.09 ± 0.36[d]	30.35 ± 1.86[c]	14.81 ± 0.46[d]
n-3/n-6	1.23 ± 0.10[c]	2.81 ± 0.05[d]	0.86 ± 0.06[c]	2.45 ± 0.32[d]
MUFA/PUFA	0.51 ± 0.11[c]	0.48 ± 0.04[a]	0.30 ± 0.05[b]	0.46 ± 0.06[b]
UFA/SFA	2.64 ± 0.30	2.93 ± 0.22	2.90 ± 0.46	3.04 ± 0.26
UI	234.82 ± 18.35[c]	262.53 ± 8.77[d]	255.32 ± 14.94[c]	265.59 ± 18.50[d]

[a,b]Significant effect ($P < 0.05$) of the rearing temperature between a and b inside one diet (Mann-Whitney test); [c,d]significant effect ($P < 0.05$) of the diet between c and d inside one rearing temperature (Mann-Whitney test).
[a]Values are means of three to four pools ± SE (molar %) of six to nine fish sperm.
[b]See Table 4 for abbreviations.

monolayer. This distribution was determined by their properties of transmembrane movement. For a given sample, the apparent correlation times calculated from the ESR spectra revealed that the higher the recording temperature, the lower the membrane microviscosity surrounding the spin label. The rearing temperature, however, did not affect membrane viscosity. The correlation times recorded at a given temperature (8 or 18°C) were the same for fish reared at 8 or 18°C (Fig. 1). This means that no adaptation of spermatozoan membranes of cold-acclimated trout compensated for the cold-induced increase in viscosity. Dietary lipids also had no effect on plasma membrane microviscosity as assessed by ESR. In addition, for any sperm cell sample, the microviscosity of the inner monolayer was always lower than that of the outer monolayer since the apparent correlation time of SL-PS was shorter than that of SL-PC.

DISCUSSION

Plasma membrane characteristics. Teleostean spermatozoa have no acrosome, and fertilization of the eggs occurs after the spermatozoa have passed through the micropylar canal to reach the egg plasma membrane. Prior to this study, no information was available on the phospholipid composition of the plasma membranes of such spermatozoa. Our results show that by comparison with mammals (33–37), levels of sphingomyelin in trout spermatozoan plasma membrane are very low. This low sphingomyelin content was also observed in trout erythrocyte plasma membranes (2.8%) (38). Phosphatidylserine levels, on the contrary, are higher in trout spermatozoan plasma membrane than in mammalian ones. The distribution of phosphatidylcholine and phosphatidylethanolamine in trout spermatozoan membrane is quite similar to that found in vertebrate membranes of other cell types, where these classes predominate. Phosphatidylethanolamine is more abundant, however, in trout spermatozoa than in mammalian ones. One could expect that the high phosphatidylethanolamine levels in trout plasma membranes would contribute to enhancing their fusion capacity as unsaturated phosphatidylethanolamines are thought to show a tendency toward forming nonbilayer-type configurations due to their "cone-shaped" structure (39,40). Surprisingly, we observed

TABLE 6

Fatty Acid Composition of Phosphatidylserines in Spermatozoan Plasma Membrane from Trout Reared at 8 or 18°C and Fed with Either a Corn Oil-Supplemented or a Cod Liver Oil-Supplemented Diet Before Spawning[a]

Fatty acid[b]	8°C Reared		18°C Reared	
	Corn oil diet	Cod liver oil diet	Corn oil diet	Cod liver oil diet
14:0	1.30 ± 0.10[a]	1.43 ± 0.70[a]	1.94 ± 0.25[b]	2.09 ± 0.33[b]
15:0	0.69 ± 0.04[a]	0.90 ± 0.35[a]	1.15 ± 0.20[b]	1.40 ± 0.16[b]
16:0	8.18 ± 1.47[a]	9.76 ± 2.78[a]	9.65 ± 1.92[b]	10.86 ± 2.27[b]
17:0	0.32 ± 0.03[a]	0.39 ± 0.05[a]	0.37 ± 0.05[b]	0.45 ± 0.14[b]
18:0	7.50 ± 1.90	7.20 ± 1.34	7.87 ± 1.14	8.18 ± 1.43
16:1n-9	2.69 ± 0.93[a]	3.03 ± 0.98[a]	3.64 ± 1.06[b]	3.47 ± 2.25[b]
16:1n-7	0.24 ± 0.21	0.47 ± 0.13	0.42 ± 0.10	0.41 ± 0.28
18:1n-9	27.58 ± 2.04	24.75 ± 1.35	25.36 ± 3.84	24.04 ± 0.38
18:1n-7	0.99 ± 0.06[c]	1.26 ± 0.12[d]	0.80 ± 0.19[c]	1.41 ± 0.09[d]
20:1n-9/7	0.45 ± 0.22	0.39 ± 0.21	0.25 ± 0.08	0.54 ± 0.35
18:2n-6	6.28 ± 0.96[c]	7.50 ± 1.60[c]	4.69 ± 0.98[c]	8.14 ± 0.45[d]
18:3n-3	0.61 ± 0.57	0.45 ± 0.32	0.34 ± 0.24	0.71 ± 0.45
20:2n-6	0.89 ± 0.76[c]	0.24 ± 0.09[d]	0.74 ± 0.13[c]	0.35 ± 0.21[d]
20:3n-6	0.87 ± 0.16[c]	0.26 ± 0.11[d]	1.22 ± 0.19[c]	0.42 ± 0.29[c]
20:4n-6	5.33 ± 1.45[c]	3.08 ± 1.06[d]	7.10 ± 1.41[c]	3.03 ± 0.45[d]
20:5n-3	4.38 ± 1.33[c]	6.68 ± 1.57[d]	3.53 ± 0.99[c]	6.19 ± 0.24[d]
22:3n-9	0.23 ± 0.12	0.25 ± 0.20	0.18 ± 0.12	0.07 ± 0.07
22:5n-6	0.75 ± 0.23[c]	0.46 ± 0.25[d]	1.35 ± 0.11[c]	0.41 ± 0.12[d]
22:5n-3	0.99 ± 0.20[c]	1.76 ± 0.73[d]	0.94 ± 0.04[c]	1.25 ± 0.86[d]
22:6n-3	24.74 ± 0.72	25.86 ± 3.00	24.58 ± 2.85	23.39 ± 2.65
Sum SFA	18.73 ± 3.04[a]	20.16 ± 3.16[a]	24.48 ± 3.43[b]	23.91 ± 2.63[d]
Sum MUFA	35.20 ± 4.07[a,c]	32.40 ± 1.81[a,d]	32.91 ± 4.01[b,c]	30.96 ± 2.18b,[d]
Sum PUFA	46.07 ± 1.09	47.45 ± 3.24	45.61 ± 2.29	45.14 ± 4.53
Sum n-3	30.83 ± 1.61[c]	34.95 ± 2.62[d]	29.44 ± 2.20[c]	31.73 ± 3.74[d]
Sum n-6	14.81 ± 0.47[c]	11.93 ± 2.06[d]	15.70 ± 0.99[c]	12.95 ± 1.42[d]
n-3/n-6	2.08 ± 0.17[c]	2.99 ± 0.50[d]	1.88 ± 0.21[c]	2.46 ± 0.27[d]
MUFA/PUFA	0.77 ± 0.11[c]	0.69 ± 0.07[d]	0.72 ± 0.12[c]	0.69 ± 0.11[d]
UFA/SFA	4.44 ± 0.97[a]	4.04 ± 0.69[a]	3.74 ± 0.71[b]	3.22 ± 0.46[b]
UI	258.09 ± 8.15	265.49 ± 17.72	257.01 ± 10.60	246.56 ± 21.75

[a,b]Significant effect ($P < 0.05$) of the rearing temperature between a and b inside one diet (Mann-Whitney test); [c,d]significant effect ($P < 0.05$) of the diet between c and d inside one rearing temperature (Mann-Whitney test).

[a]Values are means of three to four pools ± SE (molar %) of six to nine fish sperm.

[b]See Table 4 for abbreviations.

that trout spermatozoan plasma membranes contain about 1% of diphosphatidylglycerol. This phospholipid is thought to be almost exclusively localized in the inner mitochondrial membrane (41). Such a contamination in our plasma membrane fraction is unlikely since no succinic dehydrogenase (mitochondrial marker) activity was detected (22). A mitochondrial contamination leading to add 1% of diphosphatidylglycerol to the total plasma membrane phospholipids would surely be detectable by enzymatic marker assay. We can then reasonably assume that trout spermatozoan plasma membrane contains diphosphatidylglycerol. In mammals, some authors have reported its presence in plasma membranes of ram epididymal or ejaculated spermatozoa (42,37).

Of the four phospholipid classes studied here, phosphatidylcholine was the most saturated, due to very high levels of 16:0. Such a preeminence of 16:0 in phosphatidylcholine is often described, whether in whole cells or in plasma membrane. Examples include cod (*Gadus morhua*) erythrocytes (43), cod and trout muscle, liver, gill, heart, kidney, and spleen (44,45), buffalo fish (*Jotiobus cyprinellus*) sperm (46), and brush border membrane from rainbow trout intestine (20). In the present study, phosphatidylinositol also displayed very saturated profiles as compared to phosphatidylethanolamine and phosphatidylserine, which contained more PUFAs. The plasma membranes of mammalian spermatozoa differ markedly from trout spermatozoan membranes in regard to fatty acid saturation in the various classes of phospholipids. In goat and boar caudal epididymis, phosphatidylethanolamine fatty acids are more saturated than those of phosphatidylcholine (35,34). In these species, furthermore, phosphatidylinositol and phosphatidylserine are the most saturated phospholipids (SFA percentages for boar caudal epididymis are respectively 98 and 64%). In phosphatidylinositol of trout spermatozoan plasma membrane, arachidonic and stearic acids predominate. This is also true in trout gill, spleen, and kidney (47) and in mammals. Phosphatidylinositol may be the primary source of arachidonate for lipoxygenase and cyclooxygenase reactions in fish sperm as postulated for gonad cell lines (48), since prostaglandins have been identified in the testes and milt of numerous fish, including trout (14,15).

Dietary effects. The dietary fats affected the various phospholipid classes differently. The n-3/n-6 ratio was much less affected in phosphatidylserine (1.5-fold) than in the other phospholipids (2.5 to 2.7-fold). This was due, however, to the

TABLE 7

Fatty Acid Composition of Phosphatidylinositols in Spermatozoan Plasma Membrane from Trout Reared at 8 or 18°C and Fed with Either a Corn Oil-Supplemented or a Cod Liver Oil-Supplemented Diet Before Spawning[a]

Fatty acid[b]	8°C Reared		18°C Reared	
	Corn oil diet	Cod liver oil diet	Corn oil diet	Cod liver oil diet
14:0	1.36 ± 0.40	2.49 ± 0.57	1.54 ± 0.92	1.53 ± 0.69
15:0	0.09 ± 0.02	0.17 ± 0.05	0.10 ± 0.05	0.09 ± 0.05
16:0	11.20 ± 1.13	15.25 ± 2.04	10.76 ± 2.47	12.67 ± 2.94
17:0	0.68 ± 0.25	0.94 ± 0.14	0.64 ± 0.11	0.81 ± 0.08
18:0	19.16 ± 1.95[a]	19.12 ± 2.35[a]	23.79 ± 3.13[b]	24.32 ± 0.25[b]
16:1n-9	2.25 ± 0.44	4.04 ± 1.57	2.81 ± 1.40	2.91 ± 1.58
16:1n-7	0.20 ± 0.24	0.58 ± 0.37	0.39 ± 0.27	0.39 ± 0.42
18:1n-9	21.97 ± 1.10[c]	15.10 ± 1.86[d]	17.38 ± 0.96[c]	12.87 ± 3.77[d]
18:1n-7	1.12 ± 0.22	1.17 ± 0.25	0.80 ± 0.19	1.17 ± 0.12
20:1n-9/7	0.34 ± 0.29	0.27 ± 0.18	0.20 ± 0.11	0.31 ± 0.15
18:2n-6	6.27 ± 0.92[c]	3.63 ± 0.61[d]	5.27 ± 0.92[c]	3.66 ± 0.67[d]
18:3n-3	1.13 ± 0.97	1.06 ± 0.73	0.56 ± 0.34	0.62 ± 0.18
20:2n-6	0.91 ± 0.77	0.32 ± 0.18	0.41 ± 0.16	0.27 ± 0.08
20:3n-6	0.88 ± 0.43[c]	0.22 ± 0.19[d]	1.43 ± 0.22[c]	0.44 ± 0.11[d]
20:4n-6	13.22 ± 1.68[c]	8.75 ± 0.76[d]	13.43 ± 1.12[c]	7.28 ± 0.87[d]
20:5n-3	6.43 ± 2.33[c]	13.18 ± 2.18[d]	7.38 ± 1.55[c]	14.86 ± 1.39[d]
22:3n-9	0.20 ± 0.15	0.25 ± 0.14	0.21 ± 0.07	0.30 ± 0.05
22:5n-6	0.61 ± 0.44[c]	0.21 ± 0.24[d]	0.27 ± 0.06[c]	0.16 ± 0.04[d]
22:5n-3	0.74 ± 0.59	0.30 ± 0.15	0.21 ± 0.05	0.38 ± 0.06
22:6n-3	6.78 ± 0.65	8.11 ± 1.55	7.78 ± 1.04	9.29 ± 1.42
Sum SFA	32.93 ± 1.49	38.50 ± 1.91	37.30 ± 2.57	39.97 ± 3.60
Sum MUFA	28.59 ± 0.75[c]	24.84 ± 2.55[d]	24.99 ± 3.32[c]	22.30 ± 1.82[d]
Sum PUFA	38.47 ± 1.51	36.67 ± 2.72	37.70 ± 2.99	37.73 ± 1.82
Sum n-3	15.25 ± 1.25[c]	22.68 ± 3.44[d]	15.94 ± 2.64[c]	25.19 ± 2.66[d]
Sum n-6	22.77 ± 1.94[c]	13.63 ± 1.00[d]	21.20 ± 0.40[c]	12.12 ± 1.02[d]
n-3/n-6	0.68 ± 0.10[c]	1.69 ± 0.38[d]	0.75 ± 0.11[c]	2.10 ± 0.40[d]
MUFA/PUFA	0.74 ± 0.04[a]	0.68 ± 0.11[a]	0.67 ± 0.13[b]	0.59 ± 0.02[b]
UFA/SFA	2.04 ± 0.14[a]	1.60 ± 0.12[a]	1.69 ± 0.20[b]	1.52 ± 0.24[b]
UI	185.33 ± 6.95	191.13 ± 13.43	184.74 ± 14.73	197.32 ± 12.25

[a,b]Significant effect ($P < 0.05$) of the rearing temperature between a and b inside one diet (Mann-Whitney test);[c,d]significant effect ($P < 0.05$) of the diet between c and d inside one rearing temperature (Mann-Whitney test).

[a]Values are means of three to four pools ± SE (molar %) of six to nine fish sperm.

[b]See Table 4 for abbreviations.

constant high level of 22:6n-3 in this phospholipid, irrespective of the diet. This persistence of 22:6n-3 raises questions as to its physiological importance for trout spermatozoa. Yu and Sinhuber (49) showed that an EFA-deficient diet in trout induced only a 30% decrease in 22:6n-3 content of body phospholipids over a 3.5-month period. Casteldine and Buckley (32,50) reported the same phenomenon, i.e., maintenance of the 22:6n-3 levels in trout that received diets devoid of EFAs. This could be linked to slow catabolism of long-chain PUFAs or to an efficient system for recycling 22:6n-3 during lipid metabolism (32). The rate of ß-oxidation of 22:6n-3 in the mitochondria of different fish tissues is limited or null (15). This, coupled with the apparent retention of 22:6n-3 during lipid mobilization, might ensure that this fatty acid is specifically retained to fulfill its role as a major constituent of biomembrane phospholipids (15). In whole trout spermatozoa, however, Watanabe *et al.* (16) have shown that when 18:2n-6 was available in an (n-3)-deficient diet, a 50% drop in 22:6n-3 was compensated by a 4-fold increase in 20:4n-6 and 22:5n-6. Whole spermatozoa from trout fed an EFA-deficient diet for one year displayed a decrease in 22:6n-3 levels although these levels were maintained in erythrocytes (17). It has been proposed that this PUFA could play a role in controlling membrane fluidity (51) or could be a substrate of lipoxygenases (15). Our study suggests a major role for 22:6n-3 in the phospholipids of trout spermatozoan plasma membrane, since levels remain constant even when most of the available fatty acids in the diet are of the n-6 series.

Temperature effect. The temperature at which the fish were reared appears to affect neither the cholesterol and phospholipid contents of spermatozoan plasma membrane nor its phospholipid class distribution. Maintenance of cholesterol levels was somewhat surprising, considering that a correlation between cholesterol content and temperature is often described in membranes of poikilothermic animals (13). However, most of these studies dealt with short-term adaptation of the fish (less than one month), and mitochondria were the structures described to be most commonly affected by temperature. Membranes of cold-adapted poikilotherms usually possess higher proportions of phosphatidylethanolamine and, less commonly, lower proportions of phosphatidylcholine than membranes formed at higher growth temperatures. This increases the proportion of nonbilayer-forming lipids at cold temperatures. Like the cholesterol decrease, this adaptation is

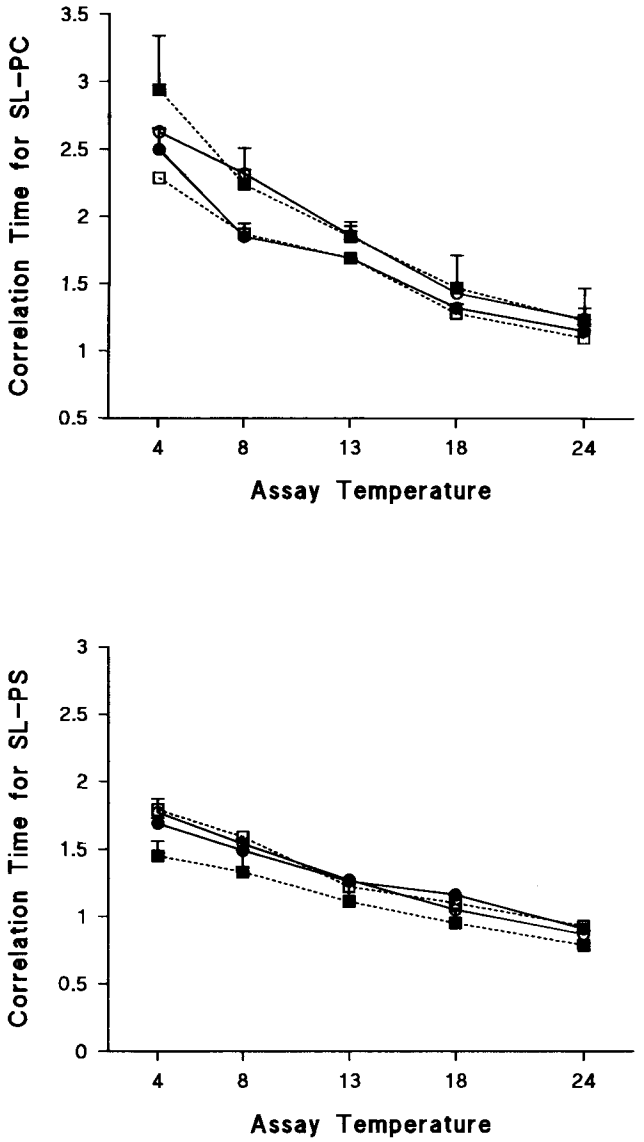


FIG. 1. Correlation time (in ns) as a function of temperature (in °C) for spin labeled-phosphatidylcholine (SL-PC) and -phosphatidylserine (SL-PS) incorporated in trout spermatozoan plasma membrane; ○, 8°C-reared trout fed with corn oil diet; ●, 8°C-reared trout fed with cod liver oil diet; □, 18°C-reared trout fed with corn oil diet; ■, 18°C-reared trout fed with cod liver oil diet. Values reported are the result of two different pools (± SE) of five to eight individual sperms. No significant differences appeared between the two diets and the two rearing temperatures.

believed to maintain a constant viscosity of the membrane. From the insignificant variations obtained for each phospholipid class in spermatozoan plasma membranes from trout reared at 8 or 18°C, it appears that this type of adaptation to low temperature did not operate in these cells. This lack of change with long-term adaptation to a different temperature is in agreement with previous observations on flounder (*Platichthys fletus*) erythrocyte plasma membranes (52). If changes in the cholesterol and phospholipid contents are merely short-term responses to thermal stress, the fact that the fish were transferred to the colder temperature prior to spermatogenesis and remained at that temperature until the process was complete could explain the apparent lack of thermal adaptation in trout spermatozoa.

A nearly ubiquitous response to exposure to a cold environment, as judged from most studies, is a drop in the proportion of SFAs and a corresponding rise in the proportion of UFAs. The introduction of double bonds in the phospholipid fatty acids disturbs the molecular packing of the membrane. Upon adaptation to a warmer temperature, conversely, an increase in the saturation level of fatty acids increases membrane viscosity. This "homeoviscous adaptation" (53) is thought to maintain a constant fluidity in biological membranes whatever the rearing temperature, in order to maintain membrane functions. We, conversely, have shown that long-term adaptation of trout to two very different rearing temperatures has little effect on the phospholipid fatty acids of trout spermatozoan plasma membranes. This is at variance with most studies on short-term adaptation of fish tissues (13): trout hepatocytes, liver plasma membranes, and mitochondria, carp liver and muscle mitochondrial membranes, and cod and carp erythrocytes (43,54). In all these cases, production of UFAs was stimulated by a sharp drop in the rearing temperature. A long-term adaptation of the fish does not always result in an acclimatory response. Although a long-term temperature adaptation resulted in slight but significant changes of the fatty acid composition in flounder erythrocyte plasma membrane (45,52), it was not the case in Atlantic salmon (*Salmo salar*) liver (55). The margin of 10°C between the two acclimation temperatures employed in our study is somewhat smaller than is commonly employed in such experiments (margins of 15 to 20°C). However, Malak *et al.* (56) showed that a six-week acclimation of trout to 11 and 21°C notably affected the fatty acid pattern of several tissues. This allows us to think that the rearing temperatures we used are of sufficient importance to trigger an acclimation response in trout. The *in vitro* experiments on trout and carp hepatocytes have shown that prolonged exposure to cold diminished the capacity for UFA production and that the total capacity to produce saturated or unsaturated species clearly did not correlate with growth temperatures (57). These observations could be one explanation for the apparent lack of response to a long-term temperature adaptation we observed in trout sperm.

The viscosity of trout spermatozoan plasma membranes as assessed by ESR appeared unaffected by the rearing temperature of the broodstock. In a previous paper (58), we showed that phosphatidylcholine is located mainly on the outer leaflet of the plasma membrane while most of the phosphatidylserine is inside. The two spin-labels used here should thus provide information on microviscosity in each bilayer leaflet. That the viscosity of neither leaflet is affected by the rearing temperature of the broodstock is in good agreement with the very slight variations observed in the fatty acid profile of each phospholipid class. Such a lack of a homeoviscous adaptation has been described for the sarcoplasmic reticulum of goldfish white muscle (59) and for the brush border membrane of carp intestinal mucosa (60). Homeoviscous adaptation is not a

property of all cellular membranes, but occurs selectively in situations where compensations maintaining membrane order serve to maintain membrane function and hence, to some extent, tissue function as well (60). The trout spermatozoan is a highly differentiated cell, but one whose metabolic activity is low and whose viability in the genital tract before spawning does not seem to depend on the molecular order of its plasma membrane. Membrane viscosity assessed by ESR with spin-labeled phospholipid gives information at the interfacial level of the membrane, since the doxyl group is located at the third carbon level. Adjustments of viscosity in the hydrophobic core cannot be detected by this probe. Hazel *et al.* (61) observed in trout hepatic plasma membranes that compensatory fluidity changes in response to temperature are more important in the hydrophobic moiety of the membrane than at the interface.

In conclusion, the fatty acid profile of phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols, and phosphatidylserines in trout spermatozoan plasma membranes was affected by the fatty acid profiles of the broodstock diet. This indicates the ability of the plasma membrane to incorporate most available fatty acids. This ability is, however, limited by the need for n-3 fatty acids as shown by the higher n-3/n-6 ratio in the plasma membrane than in the food pellets and by the constancy of the 22:6n-3 levels. As judged from the results of long-term adaptation to two different temperatures before and during spermatogenesis, the rearing temperature had little effect on the lipid composition of the plasma membranes of mature spermatozoa. This agrees with the fact that no difference in membrane viscosity was observed between the two groups and supports the hypothesis that rainbow trout spermatozoa do not display any homeoviscous adaptation.

ACKNOWLEDGMENTS

This work was supported by the Institut National de la Recherche Agronomique, by the Centre National de la Recherche Scientifique (URA 256), and by a grant from the "Région Bretagne" (Développement de Biotechnologies appliquées à l'aquaculture). K. Müller was a recipient of a short-term EMBO fellowship. We are grateful to Drs. G. Corraze, L. Frémont and X. Pelletier for kind and helpful discussions and advice. We thank Mr. Monasz for care with the fish and Mrs. Heydorff for skillful assistance.

REFERENCES

1. Malejac, M.L., Loir, M., and Maisse, G. (1990) *Aquat. Living Resour. 3*, 43–54.
2. Quinn, P.J., and White, I.G. (1967) Aust. J. Biol. Sci. 20, 1205–1215.
3. Darin-Bennett, A., and White, I.G. (1977) *Cryobiology 14*, 466–470.
4. Ansah, G.A., and Buckland, R.B. (1982) *Poult. Sci. 61*, 623–637.
5. Poulos, A., Darin-Bennett, A., and White, I.G. (1973) *Comp. Biochem. Physiol. 46B*, 541–549.
6. Parks, J.E., and Lynch, D.V. (1992) *Cryobiology 29*, 255–266.
7. Ehringer, W.D., Belcher, D., Wassall, S.R., and Stillwell, W. (1991) *Chem. Phys. Lipids 57*, 87–96.
8. Stillwell, W., Ehringer, W., and Jenski, L.J. (1993) *Lipids 2*, 103–108.
9. Salte, R., Thomassen, M.S., and Wold, K. (1988) *Bull. Eur. Ass. Fish Pathol. 8*, 63–66.
10. Hagve, T.A., Johansen, Y., and Christophersen, B. (1991) *Biochim. Biophys. Acta 1084*, 251–254.
11. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
12. Quinn, P.J., Joo, F., and Vigh, L. (1989) *Prog. Biophys. Mol. Biol. 53*, 71–103.
13. Hazel, J.R., and Williams, E.E. (1990) *Prog. Lipid Res. 29*, 167–227.
14. Greene, H.S.D., and Selivonchick, D.P. (1987) *Prog. Lipid Res. 26*, 53–85.
15. Henderson, R.J., and Tocher, D.R. (1987) *Prog. Lipid Res. 26*, 281–347.
16. Watanabe, T., Takeushi, T., Saito, M., and Nishimura, K. (1984) *Bull. Jap. Soc. Scient. Fish. 50*, 1207–1215.
17. Leray, C., and Pelletier, X. (1985) *Aquaculture 50*, 51–59.
18. Mourente, G., and Odriozola, J.M. (1990) *Fish Physiol. Biochem. 8*, 93–101.
19. Di Costanzo, G., Duportail, G., Florentz, A., and Leray, C. (1983) *Molecular Physiology 4*, 279–290.
20. Pelletier, X., Duportail, G., and Leray, C. (1987) *Membrane Biochem. 7*, 55–66.
21. Behar, D., Cogan, U., Viola, S., and Modaky, S. (1989) *Lipids 24*, 737–742.
22. Labbe, C., and Loir, M. (1991) *Fish Physiol. Biochem. 9*, 325–338.
23. Folch, J., Lees, M., and Stanley, G.M.S. (1957) *J. Biol. Chem. 226*, 497–509.
24. Bartlett, G.R. (1959) *J. Biol. Chem. 254*, 466–468.
25. Linard, A. (1988) *Cah. Techn. INRA 20*, 41–48.
26. Berry, J.F., Cevallos, W.H., and Wade, R.R. (1965) *J. Am. Oil Chem. Soc. 42*, 492–500.
27. Fellmann, P., Zachowski, A., and Devaux, P. (1994) in *Methods in Molecular Biology* (Graham, J.M., and Higgins, J.A., eds.) Vol. 27 II, pp. 161–175, Humana Press Inc., Totowa.
28. Billard, R., and Jalabert, B. (1974) *Ann. Biol. Anim. Biochim. Biophys. 14*, 601–610.
29. Keith, A., Bulfield, G., and Snipes, W. (1970) *Biophys. J. 10*, 618–629.
30. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klelk, D.C. (1985) *Anal. Biochem. 150*, 76–85.
31. Schwartz, D. (1986) *Collection Statistique en Biologie et en Médecine*, 3rd edn., pp. 246–247, Flammarion Medecine Sciences, Paris, France.
32. Casteldine, A.J., and Buckley, J.T. (1982) *Comp. Biochem. Physiol. 71B*, 119–126.
33. Parks, J.E., Arion, J.W., and Foote, R.H. (1987) *Biol. Reprod. 37*, 1249–1258.
34. Nikolopoulou, M., Soucek, D.A., and Vary, J.C. (1985) *Biochim. Biophys. Acta 815*, 486–498.
35. Rana, A.P.S., Majumber, G.C., Misra, S., and Ghosh, A. (1991) *Biochim. Biophys. Acta 1061*, 185–196.
36. Stojanoff, A., Bourne, H., Andrews, A.G., and Hyne, R.V. (1988) *Gamete Res. 21*, 297–311.
37. Hinkovska, V.T., Dimitrov, G.P., and Koumanov, K.S. (1986) *Int. J. Biochem. 18*, 1115–1121.
38. Bolis, L., and Fange, R. (1979) *Comp. Biochem. Physiol. 62B*, 345–348.
39. Mantsch, H.H., Martin, A., and Cameron, D.G. (1981) *Biochemistry 20*, 3138–3145.
40. Lindblom, G., and Rilfors, L. (1989) *Biochim. Biophys. Acta 988*, 221–256.

41. Ioannou, P.V., and Golding, B.T. (1979) *Prog. Lipid Res. 17*, 279–318.
42. Parks, J.E., and Hammerstedt, R.H. (1985) *Biol. Reprod. 32*, 653–668.
43. Lie, O., Lied, E., and Lambertsen, G. (1989) *Aquaculture 79*, 137–144.
44. Lie, O., and Lambertsen, G. (1991) *J. Chromatogr. 565*, 119–129.
45. Sorensen, P.G. (1993) *Comp. Biochem. Physiol. 106B*, 907–912.
46. Drokin, S. (1993) *Aquat. Living Resour. 6*, 49–56.
47. Turner, M.R., Leggett, S.L., and Lumb, R.H. (1989) *Comp. Biochem. Physiol. 94B*, 575–579.
48. Tocher, D.R. (1990) *Fish Physiol. Biochem. 8*, 239–249.
49. Yu, T.C., and Sinhuber, R.O. (1972) *Lipids 7*, 450–454.
50. Casteldine, A.J., and Buckley, J.T. (1980) *J. Nutr. 110*, 675–685.
51. Farkas, T., Csengeri, I., Majoros, F., and Olah, J. (1980) *Aquaculture 20*, 29–40.
52. Sorensen, P.G. (1990) *Comp. Biochem. Physiol. 96B*, 571–576.
53. Sinensky, M. (1974) *Proc. Natl. Acad. Sci. USA 71*, 522–525.
54. Dey, I., Szegletes, T., Buda, Cs., Nemcsok, J., and Farkas, T. (1993) *Lipids 28*, 743–746.
55. Skuladottir, G.V., Schiöth, H.B., Gemundsdottir, E., Richards, B., Gardarsson, F., and Jonsson, L. (1990) *Aquaculture 84*, 71–80.
56. Malak, N.A., Brichon, G., Meister, R., and Zwingelstein, G. (1989) *Lipids 24*, 318–324.
57. Hazel, J.R. (1984) *Am. J. Physiol. 246*, R460–R470.
58. Müller, K., Labbe, C., and Zachowski, A. (1994) *Biochim. Biophys. Acta 1192*, 21–26.
59. Cossins, A.R., Christiansen, J., and Prosser, C.L. (1978) *Biochim. Biophys. Acta 511*, 442–454.
60. Lee, A.C., and Cossins, A.R. (1990) *Biochim. Biophys. Acta 1026*, 195–203.
61. Hazel, J.R., McKinley, S.J., and Williams, E.E. (1992) *J. Comp. Physiol. 162*, 593–601.

[Received February 19, 1994, and in revised form December 6, 1994; Revision accepted December 12, 1994]

ω-3 Fatty Acids in Smooth Muscle Cell Phospholipids Increase Membrane Cholesterol Efflux

Eric Dusserre*, Thérèse Pulcini, Marie-Claude Bourdillon, Maryvonne Ciavatti and François Berthezene
National Institute of Health and Medical Research, INSERM U63,
Nutrition and Vascular Pathophysiology Unit, F-69675 Bron-Lyon, France

ABSTRACT: The aim of our work was to determine whether fatty acid modifications in smooth muscle cell phospholipids affect cholesterol efflux and desorption. [^{3}H]Cholesterol was used to label cholesterol pools in the whole cell or selectively in the plasma membrane. Cells were incubated for 12 h in order to increase oleate, linoleate, arachidonate, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) in phospholipids. Cholesterol efflux was monitored using native or tetranitromethane modified high-density lipoprotein$_3$ (HDL_3). When all cholesterol pools were labeled, the efflux from cells treated with different fatty acids were not different. Plasma membrane cholesterol efflux remained unchanged after oleate, linoleate or arachidonate treatments, but was markedly increased after EPA and DHA enrichment, both with native HDL_3 and with tetranitromethane–high-density lipoprotein. These results suggest that the positive effects of n-3 fatty acid consumption on the atherosclerotic process could be linked in part to an increase in plasma membrane cholesterol efflux from vascular smooth muscle cells. *Lipids 30,* 35–41 (1995).

Aortic smooth muscle cells play a key role in the atherosclerotic process particularly by accumulating cholesterol before they are transformed into foam cells (1–3). The mechanisms of foam cell transformation remain uncertain but involve abnormalities of cholesterol transport. Cholesterol efflux is widely believed to be an important factor in regulating cell cholesterol homeostasis (4), and the rate-limiting step of this process was shown to be the desorption of cell membrane cholesterol into the culture medium (5). Cholesterol desorption involves lipid–lipid interactions between membrane cholesterol and lipoproteins, and the extraction of cholesterol from its hydrophobic environment depends on membrane physicochemical characteristics (6). Membrane physical properties and membrane fluidity have been reported to be strongly dependent on the membrane composition, particularly the phospholipid fatty acid profile (7).

The aim of this study was to induce changes in membrane phospholipid unsaturated fatty acid content in order to modify desorption and exchange of cell membrane cholesterol. Five different fatty acids were added to the media of confluent cultured aortic smooth muscle cells (SMC) and incorporated into cell membrane phospholipids during short time incubations.

ω-3 Fatty acids were of particular interest in regard to atherosclerosis prevention (8), as many studies have shown an inverse correlation between n-3 fatty acid consumption and the prevalence of coronary heart disease. The influence of fatty acid enrichment on cholesterol desorption was examined by selectively labelling the membrane cholesterol pool before efflux experiments were performed with different cholesterol acceptors.

MATERIALS AND METHODS

Cell culture. Aortic SMC were obtained from 6–10-week-old rats, as previously described (9). Briefly, the media layer of thoracic aorta was dissected out and SMC dispersed into single cells by collagenase (2 mg/mL, CLSI; Worthington Biochemical Corp., Freehold, NJ) and elastase (0.4 mg/mL, Type III; Biosys, Compiègne, France). Cells were seeded in Petri dishes (35 mm) or 6-multiwell plates ($3–5 \times 10^4$ cells/cm^2). Cells were grown in minimum Eagle's medium (MEM) supplemented with 10% newborn calf serum, penicillin/ streptomycin (100 units/mL and 50 μg/mL, respectively) and glutamine (2 mM). Medium was renewed three times a week. Secondary cultures were carried out in 25 cm^2 Falcon flasks using trypsin (0.08%). Cells were reseeded at 2.10^5 cells per flask and studied between 2 and 4 passages. For experiments, cells in Falcon flasks were used during the post-confluent phase.

Fatty acid enrichment procedure. Cells were incubated with fatty acid–bovine serum albumin (BSA) complexes during 12 h (10). The fatty acids used were oleic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), all from Sigma Chemical Corp. (St. Louis, MO).

The 50-mM fatty acid stock solutions were prepared as previously described (10) by drying fatty acids under N_2, on ice.

*To whom correspondence should be addressed at INSERM, Unit 63, 22 Ave. Doyen Lépine, CP 18, F-69675 BRON Cedex, France.

Abbreviations: BSA, bovine serum albumin; DHA, docosahexaenoic acid (22:6; 4,7,10,13,16,19); EDTA, ethylenediaminetetraacetate; EPA, eicosapentaenoic acid (20:5; 5,8,11,14,17); GLC, gas–liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MEM, medium Eagle's medium; PTA, phosphotungstic acid; PUFA, polyunsaturated fatty acid; SMC, smooth muscle cells; TLC, thin-layer chromatography; TNM, tetranitromethane.

Copyright © 1995 by AOCS Press

The dried fatty acids were dissolved in methanol containing 12.5 μg/mL butylated hydroxytoluene. MEM without calf serum containing 0.1 mM fatty acid and 2 mg BSA/mL was used for fatty acid enrichment procedures. Control medium consisted of MEM without calf serum containing 2 mg/mL BSA.

Fatty acid composition of SMC phospholipids. Lipids were extracted according to the method of Folch *et al.* (11). Fatty acid composition of cell phospholipids was studied by gas–liquid chromatography (GLC) after separation of neutral lipids by thin-layer chromatography (TLC) using a petroleum ether/diethyl oxide/acetic acid solvent system (90:30:2). Phospholipids were scraped and re-extracted as described previously (10). Phospholipid fatty acids were methylated according to the technique of Morrison and Smith (12). Methylated fatty acids were analyzed in an Intersmat IGC 120 DFL using a flame-ionization detector and a 30 m × 0.53 mm DB 23 column (J&W Scientific, Folsom, CA). The oven temperature was 128°C at injection and maintained during 10 min, then increased at a rate of 2°C/min for 6 min, and finally raised at a rate of 4°C/min up to 235°C. Injector and detector temperatures were 150 and 250°C, respectively. Helium was used as carrier gas under a pressure of 0.8 bars.

Lipoproteins and modified lipoproteins. High-density lipoproteins $_3$ (HDL_3; density range 1.125–1.21 g/mL) were isolated by precipitation and standard sequential ultracentrifugation of healthy donor human plasma (13). For 1 mL of plasma, very low density lipoprotein and low-density lipoprotein (LDL) were precipitated with 100 μL of 4% phosphotungstic acid (PTA) and 25 μL of 2 M $MgCl_2$. The supernatant fraction containing immunoglobulins and HDL was collected. Immunoglobulins were precipitated with 900 μL of 4% PTA. After a 3500 rpm centrifugation, the supernatant phase was collected and HDL were precipitated with 175 μL of 2 M $MgCl_2$. The HDL pellet was dissolved in 0.5 M Na_2CO_3. The density was adjusted to 1.125 for a 24 h ultracentrifugation, and the HDL_2 and HDL_3 fractions were separated. The density of the infranatant containing HDL_3 fraction was adjusted to 1.21, and HDL_3 were then re-isolated by flotation after a further 24 h ultracentrifugation. Lipoproteins were dialyzed for 48 h in standard buffer with 150 mM NaCl and 10 mM ethylenediaminetetraacetate (EDTA). Protein amounts were determined by the method of Bradford (14). HDL_3 were treated with 3 mM tetranitromethane (TNM) (15) (Sigma). A stock solution of 3 M of TNM was made at room temperature in 95% ethanol. The reaction mixture included 3 mM of TNM, 150 mM NaCl and 5 mM EDTA. The incubation was performed during 1 h at room temperature with the 0.5–1.5 mg/mL HDL_3 solution. The TNM–HDL were isolated by gel filtration chromatography on Sephadex G25 column (Pharmacia, Uppsala, Sweden) and extensively dialyzed. Apolipoprotein E containing HDL_3 were eliminated by the PTA/$MgCL_2$ precipitation procedure. Apolipoprotein profiles of HDL were controlled by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15) using the Mini Protean Cell System (Biorad, Richmond, CA). Native and modified lipoprotein sizes were determined by PAGE in 4–30% native polyacrylamide gels (Pharmacia-LKB, Uppsala, Sweden). The identical migration observed excluded possible aggregation.

Labeling of intracellular cholesterol pools before fatty acid incubations. The different intracellular cholesterol pools were labeled (16) before fatty acid enrichment by incubating the cells 48 h with MEM containing 10% newborn calf serum, without previous cholesterol depletion of the cells. The calf serum was previously labeled 24 h with 5 μCi/mL of [^{3}H]cholesterol (specific activity 48.4 Ci/mmole) without lecithin cholesterol acyl transferase (LCAT) inhibition in order to allow esterification of radiolabeled cholesterol in the LDL fraction. Using this procedure, [^{3}H]cholesterol was allowed to equilibrate within the different cellular pools but was predominantly recovered within the intracellular pools. After the labeling period, 60% of the cell radioactivity was recovered in the intracellular pool, 14% in the plasma membrane and 26% in the cholesteryl ester fraction, as assessed by the cholesterol oxidase procedure (see below). The degree of [^{3}H]cholesterol labeling was measured at the end of the fatty acid enrichment period and was taken as 100% for efflux measurements.The [^{3}H]cholesterol present in the cells at the end of the cholesterol labelling period was measured in separate dishes for each experiment. Before the fatty acid enrichment period, 83540 ± 1860 cpm were recovered in the cells. After the fatty acid enrichment period, 79120 ± 2130, 80340 ± 1860, 78350 ± 2200, 78980 ± 3170, 77520 ± 3080 and 79610 ± 2490 cpm were recovered in the cells for control, oleate-, linoleate-, arachidonate-, EPA- and DHA-treated cells, respectively. Only a slight efflux was observed during the fatty acid enrichment period, and it was the same for all the fatty acid-enriched cells. This is consistent with the finding that most of the label remains in the intracellular pools after the labeling period.

Labeling of plasma membrane cholesterol after fatty acid incubations. After fatty acid incubations as described above, SMC were incubated for 45 min at 10°C with serum free medium containing 2 μCi/mL of [^{3}H]cholesterol (Amersham, Amersham, United Kingdom) added in ethanol. With this procedure, labeled cholesterol is known to be selectively incorporated in the plasma membrane pool as previously shown (17–19). The 100% values were 30350 ± 2130, 29120 ± 2440, 32730 ± 1870, 28550 ± 2260, 30480 ± 2500 and 29890 ± 2135 cpm for control, oleate-, linoleate-, arachidonate-, EPA- and DHA-treated cells, respectively. In order to assess whether membrane cholesterol was specificaly labeled, cells were washed five times with cold Hank's buffer and fixed with 1 mL of 1% cold glutaraldehyde (10 min, 0°C). Cells were rinsed three times with cold sucrose buffer (310 mM sucrose, 0.5 mM NaH_2PO_4; pH 7.4) and were then incubated in the same buffer with 2 U of cholesterol oxidase (Boehringer, Ingelheim, Germany) for 2 h at 37°C. Cells were washed three times with cold Hank's buffer and then scraped with a rubber policeman. The cell suspension was collected, and lipids were extracted with 25 mL of hexane/isopropanol (3:2) and 5 mL 88% KCl 0 for 12 h. The lipid phase was dried under N_2, and the labeled lipids were separated in TLC (20 ×

20 Gel silica plates; Merck, Darmstadt, Germany) with chloroform/methanol (100:2). Cholesterol, cholestenone and cholesteryl esters were identified using the appropriate standards. Each silica gel spot was removed, and the radioactivity of each lipid fraction was quantitated by liquid scintillation counting. Under these conditions, more than 95% of cholesterol was selectively oxidized to cholestenone.

Competitive binding studies. Binding assays were carried out at 4°C in order to prevent internalization of HDL_3 or TNM–HDL. Cells were incubated for four hours with 20 μg/mL ^{125}I-HDL_3 labeled by the iodine monochloride procedure of MacFarlane (20). Briefly, 4 mg of native HDL_3 was added to glycine buffer 1M in NaCl 0.9% and mixed with 1 mCi NaI (10 μL) + 13 μL ICl (from a stock solution 0.033 M) added to 200 μL glycine buffer 1 M in NaCl 0.9%. HDL_3 was separated by gel filtration chromatography on Sephadex G25 column. ^{125}I-HDL_3-labeled cells were pulse-chased by 100, 200 and 300 μg of unlabeled HDL_3 or TNM–HDL in MEM without calf serum medium. Cells were washed three times with NaCl 0.9%/(2-amino-2-hydroxymethylpropane-1,3-diol)/BSA and three times with the same buffer without BSA. In order to assess the influence of fatty acid preincubations on the number of HDL_3 high affinity binding sites expressed by the cells, competitive binding experiments were performed between fatty acid preincubated cells and control cells using the same procedure as described earlier.

Efflux assays. After the fatty acid incubations and the labeling procedure, cells were rinsed five times with cold Hank's buffer and 200 μg/mL of HDL_3 or TNM–HDL. Media conditions for control cells were identical as for treated cells except that lipoproteins were replaced by 200 μg/mL of BSA. Cells were incubated at 37°C. At each indicated time, the culture medium was collected, centrifuged and counted in 8 mL of scintillating liquid (Beckmann, Fullerton, CA). Data are expressed as the percentage of the initial cell [^{3}H]cholesterol radioactivity (after fatty acid enrichment period) per well appearing in the medium. Phospholipid fatty acid composition was measured in trypsinized cells by GLC after lipid extraction with 25 mL of dichloromethane/methanol (2:1) and 5 mL KCl 0.88% overnight and phospholipid separation by TLC as described previously.

Cellular cholesterol and protein assays. Cellular cholesterol was assayed according to the colorimetric method of Omodeo Sale (21). Cellular protein content was determined as previously described by Lowry (22).

Statistical analysis. A one-way analysis of variance was used to compare values obtained under different experimental conditions, followed by pair-wise comparisons among means using the unpaired Student's *t*-test.

RESULTS

SMC fatty acid phospholipid composition after preincubation with the different free fatty acids. Table 1 shows the fatty acid profile of confluent SMC after the different fatty acid treatments. Preincubation with oleic acid (18:1n-9) resulted in a 7% increase in oleic acid content in the phospholipid fraction (40.77 ± 1.64 vs. 33.27 ± 2.52 for control cells). No major change was observed in the other fatty acids. The percentage of total monounsaturated fatty acids increased in oleic treated cells vs. control cells (46.68 ± 1.44 vs. 41.12 ± 1.56). The percentage of n-9 fatty acids increased significantly (10%): 47.57 ± 1.96 vs. 37.8 ± 2.67 in control cells.

Linoleic acid (18:2n-6) treatment led to a twofold increase in linoleic acid content (9.73%) in treated cells and a slight increase in the polyunsaturated fatty acids (PUFA) percentage (21.82 ± 1.56 vs. 17.91 ± 0.61) and in n-6 content (14.75 ± 0.92 vs. 12.45 ± 0.85).

Arachidonic acid (20:4n-6) treatment gave the highest percentage in phospholipid arachidonic acid (13.1 ± 1.71 vs. 3.51 ± 0.57 in controls), in PUFA acids (26.73 ± 1.30 vs. 17.91 ± 0.61) and in n-6 fatty acids (20.06 ± 1.26 vs. 12.45 ± 0.85 in controls).

EPA (20:5n-3) incubations resulted in 7.94 ± 1.52 in EPA vs. 2.46 ± 0.33 in control cells, 26.23 ± 4.53 in PUFA vs. 17.91 ± 0.61 in controls, 18.6 ± 5.03 in n-3 fatty acids vs. 4.71 ± 0.62 in controls.

DHA (22:6n-3) treatment produced 9.55 ± 0.73% DHA (vs. 0.62 ± 0.17 for control cells) in phospholipids, 22.84 ± 0.82 (vs. 17.91 ± 0.61 for control cells) in PUFA and 16.74 ± 2.97 in n-3 fatty acids vs. 4.71 ± 0.62 in controls.

Measurement of unsaturation in each group of treated cells was expressed using an unsaturation index calculated as follows (10):

$$\text{unsaturated index} = \sum(\%\ \text{of each fatty acid} \times \text{amount of double bonds per fatty acid}) \quad [1]$$

According to this formula, both EPA- and DHA-treated cells showed a significant increase in unsaturation index (171.05 and 140.03, respectively) as compared to control cells or to other fatty acid treatments. In summary, the five fatty acid treatments resulted in five different SMC phospholipid profiles, characterized by a different PUFA percentage and n-3 percentage.

HDL_3 and TNM-modified HDL-mediated efflux. Efflux of cell cholesterol was measured using two different protocols. A: The intracellular cholesterol pools were labeled by incubating confluent cells during 48 h with [^{3}H]cholesterol radiolabeled MEM before fatty acid enrichment procedure because fatty acid incubations were shorter in time in regard to cholesterol labelling procedure. If fatty acids had been incubated first, 48 h of cholesterol labeling might have allowed fatty acid transformations. Efflux experiments were performed after different incubation delays with 200 μg HDL_3/mL. No significant differences could be found in HDL_3 mediated efflux within the different fatty acid treated cells (data not shown). B: Plasma membrane cholesterol was selectively labeled after prior incubations with the different fatty acids. Before incubation with the lipoproteins, no difference could be observed in the amount of labeled cholesterol incorporated in the plasma membrane of the different fatty acid treated cells (data not shown).

TABLE 1
Fatty Acid Composition of Smooth Muscle Cell Phospholipids

	Treatment					
	Controls	18:1n-9	18:2n-6	20:4n-6	20:5n-3	22:6n-3
16:0	11.10 ± 1.58[a]	8.88 ± 1.27	13.21 ± 2.00	13.93 ± 1.78	10.86 ± 1.45	10.12 ± 1.56
16:1n-9	1.29 ± 0.36	O.98 ± 0.22	1.64 ± O.36	1.49 ± O.64	1.11 ± 0.26	0.67 ± 0.18
18:0 DMA	1.47 ± 0.24	1.06 ± 0.22	1.15 ± 0.22	1.10 ± 0.34	0.85 ± 0.16	1.43 ± 0.21
18:1 DMA	1.15 ± 0.30	1.19 ± 0.41	1.15 ± 0.22	0.66 ± 0.36	1.03 ± 0.24	1.20 ± 0.30
18:0	17.82 ± 1.10	16.24 ± 1.22	17.20 ± 1.69	18.44 ± 2.57	14.72 ± 1.73	15.83 ± 1.19
18:1n-9	33.27 ± 2.52	40.77 ± 1.64[b]	19.90 ± 1.60[c]	20.29 ± 2.80[c]	21.93 ± 1.81[c]	25.36 ± 3.05
18:1n-7	1.73 ± 1.10	0.09 ± 0.09	1.57 ± 1.10	0.50 ± 0.50	1.84 ± 0.67	1.50 ± 0.67
18:2n-6	4.96 ± 0.32	3.92 ± 0.36	9.73 ± 1.01[c]	3.55 ± 1.04	3.01 ± 0.31[c]	3.31 ± 0.29[c]
18:3n-3	0.40 ± 0.06	0.48 ± 0.16	0.58 ± 0.15	1.05 ± 0.40	0.57 ± 0.09	2.10 ± 1.01
20:4n-6	3.51 ± 0.57	3.35 ± 0.69	3.04 ± 0.33	13.1 ± 1.71[d]	1.96 ± 0.25[b]	2.65 ± 0.37
20:5n-3	2.46 ± 0.33	2.15 ± 0.57	3.83 ± 0.74	3.00 ± 1.07	7.94 ± 1.52[c]	1.94 ± 0.59
22:0	1.71 ± 0.27	1.86 ± 0.24	2.37 ± 1.09	0.76 ± 0.15[c]	3.64 ± 1.30	2.86 ± 0.27[b]
24:0	2.69 ± 0.82	2.12 ± 0.39	2.48 ± 0.49	3.21 ± 0.53	4.67 ± 0.35	5.95 ± 1.61
22:4n-6	1.23 ± 0.13	0.81 ± 0.11[b]	1.28 ± 0.47	2.27 ± 0.68	0.90 ± 0.29	0.53 ± 0.19[b]
22:5n-6	2.55 ± 1.16	0.70 ± 0.18	0.57 ± 0.15	0.90 ± 0.29	0.67 ± 0.19	0.61 ± 0.28
22:5n-3	1.22 ± 0.51	0.23 ± 0.10	0.24 ± 0.14	1.07 ± 0.39	0.64 ± 0.27	2.48 ± 1.96
22:6n-3	0.62 ± 0.17	0.59 ± 0.08	0.76 ± 0.27	0.91 ± 0.26	9.44 ± 4.38	9.55 ± 0.73[d]
24:1n-9	0.61 ± 0.23	1.97 ± 1.34	2.57 ± 1.99	1.73 ± 1.11	0.35 ± 0.14	0.26 ± 0.21
% PUFA	17.91 ± 0.61	13.60 ± 1.23[c]	21.82 ± 1.56[b]	26.73 ± 1.30[d]	26.23 ± 4.53	22.84 ± 0.82[d]
% Saturated	34.11 ± 0.70	30.44 ± 1.28[b]	36.21 ± 3.30	38.07 ± 1.87	34.66 ± 2.19	36.72 ± 2.62
% MUFA	41.12 ± 1.56	46.68 ± 1.44[c]	31.23 ± 2.37[c]	27.7 ± 2.85[c]	30.63 ± 2.17[c]	33.90 ± 2.09[b]
Unsat. index	105.70 ± 4.41	92.90 ± 4.70	95.94 ± 7.90	129.25 ± 4.58[c]	171.05 ± 26.0[b]	140.03 ± 10.4[c]
% n-3	4.71 ± 0.62	3.46 ± 0.57	5.43 ± 0.84	6.05 ± 0.91	18.60 ± 5.03[c]	16.74 ± 2.97[d]
% n-9	37.89 ± 2.67	47.57 ± 1.96[b]	28.09 ± 2.31[b]	25.75 ± 2.70[c]	26.46 ± 2.19[b]	30.45 ± 3.29
% n-6	12.45 ± 0.85	8.89 ± 0.86[b]	14.75 ± 0.92	20.06 ± 1.26[d]	6.73 ± 0.54[d]	10.90 ± 3.35

[a]Values are mean ± SD weight % of total fatty acids in the phospholipid fraction from six typical experiments: For one experiment, six 75 cm^2 flask of cells were used for each fatty acid pre-incubation, and the experiments were renewed six times. At the end of the pre-incubation time, the cells were trypsinized and the lipids were extracted according to Folch *et al.* (11); phospholipids were separated and samples were methylated for gas–liquid chromatography. DMA, dimethyl acetal; PUFA, polyunsaturated fatty acid; MUFA, monounsatured fatty acid; unsat., unsaturated.
[b]$P < 0.05$ vs. controls.
[c]$P < 0.01$ vs. controls.
[d]$P < 0.001$ vs. controls.

In oleic, linoleic and arachidonic acid-treated cells, no significant differences could be observed in TNM-modified HDL-mediated efflux, nor in HDL_3-mediated efflux, compared to control cells. In EPA- and DHA-treated cells, TNM- modified HDL-mediated efflux and native HDL_3-mediated efflux were greatly increased (Figs. 1 and 2)—+30% for EPA- and DHA-treated cells vs. control cells after 5 h incubation with 200 μg TNM-modified HDL; and +25% for EPA- and +35% for DHA-treated cells after 5 h incubation with 200 μg HDL_3.

Competition of ^{125}I-labeled HDL_3 binding by unlabeled native and TNM-modified HDL_3 showed that TNM-modified HDL could not displace labeled HDL_3, as native HDL_3 did (98.8 vs. 68.5% binding, with 300 μg protein/mL, respectively; Table 2). Furthermore, competition of ^{125}I-labeled HDL_3 binding by unlabeled native HDL_3 in n-3 fatty acid-treated cells and control cells showed no difference (Table 3).

Taken together, these different results indicate that the increase in plasma membrane cholesterol efflux observed in n-3 fatty acid-treated cells could not be attributed to an enhancement in the number of HDL_3 cell binding sites: TNM-modified HDL-mediated efflux, as well as HDL_3-mediated efflux, were increased in n-3 fatty acid-treated cells and n-3 fatty acid-treated cells did not present any increase in the number of high affinity binding sites for HDL_3.

Free cholesterol content of SMC after 12 h incubation with EPA and DHA. Cholesterol was assayed according to the method of Omodeo Sale (21), and protein content was determined following the method of Lowry (22). No difference in cholesterol content could be found between EPA- or DHA-treated cells and control cells after fatty acid enrichment procedures (Table 4). No difference in labeled cholesterol partitioning between cell membrane and intracellular pools could be found between EPA- or DHA-treated cells and control cells after fatty acid enrichment procedures and before cholesterol efflux experiments (95 ± 4, 94 ± 3 and 92 ± 5% of cholesterol was oxidized to cholestenone for control cells, EPA-incubated cells and DHA-incubated cells, respectively) (Table 4).

DISCUSSION

The purpose of the present work was to induce significant changes in phospholipid fatty acid profile of SMC in order to determine whether various types of unsaturated fatty acids used could affect the rate of cholesterol efflux from cells. A series of 12-h incubations with MEM without calf serum containing BSA or oleic, linoleic, arachidonic, EPA or DHA resulted in different SMC fatty acid profiles.

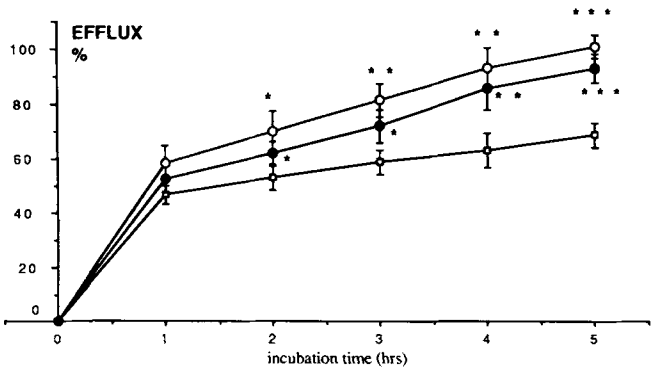


FIG. 1. Time course efflux of plasma membrane cholesterol after 12 h of incubation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and 5 h of incubation with 200 μg/mL of native high-density lipoprotein $_3$ (HDL_3). Plasma membrane cholesterol was labeled and cells were incubated with fatty acids as described. Cells were then incubated with 200 μg of native HDL_3 or with 200 μg/mL of bovine serum albumin during 5 h. The medium was collected at each indicated time. The cells were washed and trypsinized for cell counting or digested with NaOH 0.1 N in order to determine cell protein content. For the different fatty acid treatments, cell number and cell protein amounts were not significantly different. Data are values of radiolabeled cholesterol in the medium expressed as the percentage of the initial cell [^{3}H]radioactivity per well appearing in the medium. Values are means ± SD and n = 6 wells for each treatment at each time; data represent one of three identical experiments. Control (□); EPA treatment (●); DHA treatment (○). 100% value represents 30480 ± 2500 and 29890 ± 2135 cpm for EPA- and DHA-treated cells, respectively. Different from control at: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

When the intracellular cholesterol pools were labeled and the fatty acid enrichment procedure was used, no significant differences in cholesterol efflux could be detected between the different groups. The membrane enrichment in the different unsaturated fatty acids seemed to have no physical or metabolic effect on the cholesterol translocation process under our conditions. These results prompted us to specifically label the plasma membrane cholesterol pool to magnify the desorption of cholesterol from cell membrane to the medium acceptors. This step is known to be a rate-limiting factor for efflux (4,5), and the translocation from intracellular pools seemed to be unaffected by the different fatty acid incubations in our conditions. When plasma membrane cholesterol was selectively labeled, pre-incubation with n-3 fatty acids increased HDL_3-mediated cholesterol efflux and TNM–HDL-mediated cholesterol efflux. These results show that increasing EPA and DHA in cell phospholipids increased the plasma membrane cholesterol desorption from the cell membrane bilayer into the phospholipid bilayer of an acceptor particle.

As both native and TNM-treated HDL gave similar results, this process appears to involve lipid–lipid interactions rather than apoprotein-mediated cholesterol movements. Furthermore, pulse chase studies provided evidence that n-3-treated cells did not express more high affinity HDL_3 binding sites than did control cells. Taken together, these different findings clearly indicate that the increase in plasma membrane cholesterol efflux observed in n-3 fatty acid-treated cells could not be attributed to an alteration of, or enhancement in, the number of HDL_3 cell binding sites. In addition, cell cholesterol

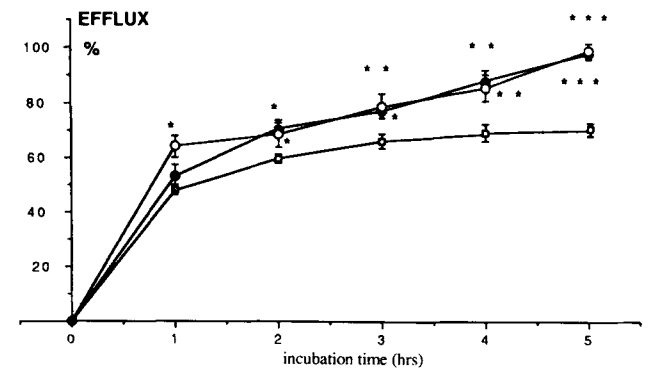


FIG. 2. Time course efflux of plasma membrane cholesterol after 12 h of incubation with EPA and DHA and 5 h of incubation with 200 μg/mL of tetranitromethane (TNM)-modified HDL. Plasma membrane cholesterol was labeled and fatty acids were incubated as previously described. Efflux of plasma membrane cholesterol was measured under the same experimental conditions as in Figure 1, except that native HDL_3 were replaced by TNM–HDL. Control (□); EPA treatment (●); DHA treatment (○). 100% value represents 30480 ± 2500 and 29890 ± 2135 cpm for EPA- and DHA-treated cells, respectively. *, **, *** and abbreviations as in Figure 1.

TABLE 2
Competition of ^{125}I-Labeled HDL_3 Binding by Unlabeled Native HDL_3 or TNM-Modified HDL[a]

Competitor concentration	% ^{125}I-labeled HDL_3 bound	
μg prot/mL	Native HDL_3	TNM-modified HDL
0	100.0 ± 2.23	100.0 ± 2.23
100	68.77 ± 4.32	97.78 ± 1.97
200	62.09 ± 1.71	100.6 ± 4.48
300	68.48 ± 1.58	98.76 ± 3.50

[a]Binding was measured with 20 μg of ^{125}I-labeled high-density lipoprotein $(HDL)_3$/dish and 0–300 μg of unlabeled native HDL_3 or tetranitromethane (TNM) modified HDL. Data are expressed as % of the amount of binding without competitors (means ± SD; six wells for each concentration). Data are from one of two similar experiments.TNM-modified HDL were unable to displace ^{125}I-labeled HDL_3 as native HDL_3 did.

TABLE 3
Competition of ^{125}I-Labeled HDL_3 Binding by Unlabeled Native HDL_3 in ω-3 Fatty Acid Treated Cells and Control Cells[a]

Competitor concentration	% ^{125}I-labeled HDL_3 bound		
μg prot/mL	Control cells	EPA-treated cells	DHA-treated cells
0	100.0 ± 2.16	100.0 ± 2.16	100.0 ± 2.16
100	56.52 ± 0.56	45.65 ± 2.11	45.53 ± 1.76
200	44.03 ± 1.10	41.22 ± 2.51	40.81 ± 1.34
300	33.37 ± 0.68	32.27 ± 1.35	34.58 ± 0.75

[a]Binding was measured with 20 μg of ^{125}I-labeled HDL_3/dish for each fatty acid pretreatment and control cells and 0–300 μg of unlabeled native HDL_3/mL. Data are expressed as % of the amount of binding without competitors (means ± SD; six wells for each concentration). Data are from one of two similar experiments. ^{125}I-labeled HDL_3 binding without unlabeled HDL_3 was identical for each fatty acid pretreated cells and control cells. The binding values were the same for ω-3 fatty acid-treated cells and control cells, for each unlabeled HDL_3 incubation. Abbreviations as in Table 2; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

TABLE 4
Free Cholesterol Distribution and Content of Smooth Muscle Cell After 12 h Incubation with Different Fatty Acids[a]

Treatment	Control cells	Oleate	Linoleate	Arachidonate	EPA	DHA
μg cholesterol/mg cell protein	39.25 ± 0.28	38.30 ± 1.5	41.60 ± 0.9	42.75 ± 1.3	40.05 ± 0.72	38.20 ± 0.13
% Cholesterol oxidizable	95 ± 4	93 ± 3	93 ± 4	95 ± 5	94 ± 3	92 ± 5

[a]Results are the mean ± SD of four independent determinations. Cholesterol was assayed according to (21) and protein content was determined by method (22). Membrane cholesterol was determined according to the cholesterol oxidase procedure. See Table 3 for abbreviations.

content was unaffected by n-3 fatty acid incubations. In contrast to the incubations with n-3 fatty acids, cells pre-incubated with oleic, linoleic or arachidonic acid had HDL_3-mediated cholesterol efflux and TNM–HDL-mediated cholesterol efflux similar to control cells.

The fatty acid incubations resulted in characteristic unsaturation patterns for each cell group (Table 1). It is difficult to determine the real efficiency of 18:1n-9 to change plasma membrane physical properties because of the relatively modest changes found. The 18:1n-9 homeostasis seems to be strongly maintained in phospholipids of SMC in culture under our conditions. Linoleic, arachidonic, EPA and DHA incubations gave a similar rise in cell PUFA percentages but provided very different patterns of fatty acid increase, suggesting that phospholipid n-3 fatty acid content is the main factor in the differences in membrane cholesterol extraction by phospholipids of acceptor particles that we found. Abundant evidence exists that dietary fatty acid consumption influences plasma lipids and lipoproteins in both human and animals (23).

Although consumption of n-3 PUFA is associated with a reduced incidence of coronary arterial diseases (8,24) and modifications of cholesterol metabolism (25,26), little is known about the influence of cellular enrichment in unsaturated fatty acids on the cholesterol metabolism of nonvascular cells (27,28). The effect of lipid modification of the membrane phospholipid pool may be to alter the conformation of proteins, which are sensitive to lipid environment (7), rather than to directly perturb bulk lipid fluidity (29) or specific lipid domains (6,30) and the physical and biochemical functions controlled by the membrane lipids.

Although effects of phospholipid fatty acid composition on cholesterol efflux rates have been studied on the human endothelial EA.hy 926 cell line (10), modifications of phospholipid fatty acids by n-3 PUFAs were studied in very few reports on SMC (31,32). Morisaki *et al.* (32), using a 16-h incubation procedure with 90 μM of 20:5n-3 and 22:6n-3 obtained results similar to ours: n-3 content was increased 1.8-fold and 2.6-fold after incubation with EPA and DHA, respectively, whereas in our study we found a 4-fold increase with EPA and 3.5-fold increase with DHA. In a previous similar work, Kilsdonk *et al.* (10) showed that in EA.hy 926 cells after cholesterol enrichment, palmitate incubation was the most efficient in increasing cholesterol efflux in an HDL_3 binding-independent manner, whereas EPA was less efficient. Such discrepancies between our results and Kilsdonk *et al.*'s (10) study could be due to differences in the two protocols: First, the two cell types used were very different. SMC were used between 2–4 passages and EA.hy 926 cells were issued from a continuous transformed cell line. Second, the efflux measurement protocols were not the same; Kilsdonk *et al.* (10) assayed cholesterol remaining in the cells after lipoprotein incubations, whereas we measured the appearance cholesterol in the medium specifically from the plasma membrane pool. Third, in our study, efflux was measured after short-time incubations with lipoproteins whereas Kilsdonk *et al.* (10) performed incubations for 12 h and more. Taking Kilsdonk *et al.*'s (10) results into account, it can be assumed that palmitate incubation in EA.hy 926 cells is more efficient for cholesteryl ester palmitate enrichment than for phospholipid palmitate enrichment and that palmitate effects on cholesterol efflux must be rather related to a modification of the different kinetic pools of membrane cholesterol. Thus, fatty acids might influence cholesterol efflux in at least two different manners—directly by incorporating into the phospholipid fraction and indirectly by affecting cholesterol distribution. On the other hand, a faster cholesterol HDL-mediated efflux was found with n-3 enriched HDL from n-3 fed rabbits (33), suggesting that n-3 in lipoproteins had a positive effect on efflux.

According to our present findings and a previous study showing that DHA increased permeability and decreased phase transition temperature of cells (34), it may be concluded that in SMC, n-3 fatty acids promoted cholesterol efflux because of the increased unsaturation they produced in the phospholipid bilayer. The increased unsaturation index found in our study might account in part for the effect of n-3 fatty acid on promoting desorption of plasma membrane cholesterol. Incorporation of EPA and DHA in phospholipids of plasma membrane increases the membrane unsaturation, much more than does oleic, linoleic or arachidonic acid incorporation. Alterations in membrane fluidity or lipid domain structures are possibly responsible for the effects observed.

The previous work of Sweet and Schroeder (35) showed that PUFA could alter transbilayer sterol localization, and it is possible that such a mechanism was involved in the enhanced plasma membrane cholesterol efflux seen with n-3 fatty acid enrichment. Mechanisms of cell cholesterol efflux remain controversial; binding site interactions are not well defined (36–38), but the fatty acid profiles of HDL (39) or cells (35,40) are greatly involved in cholesterol diffusion. The cholesterol transport through membranes involves specific pools or domains (30,41), particularly transbilayer domains within the cytofacial (inner) and exofacial (outer) leaflets. The cytofacial leaflet is known to be more rigid and contains more sterol. PUFA, incorporated into the membrane phospholipids in culture, alter the transbilayer difference in fluidity (41,42), and can result in an apparent reversal of membrane transbilayer

cholesterol distribution; more than 70% of the membrane sterol can become exofacial (35). Thus, regulation of the size of the exofacial cholesterol pool probably results in a bigger cholesterol pool available for diffusion and efflux; n-3 fatty acids in SMC phospholipids likely affect the size of this pool by a mechanism that remains to be elucidated. ω-3 Fatty acids and particularly DHA can adopt unique, closely packed arrays in lipid bilayers and thus can affect plasma membrane physical properties (43). These conformations were predicted on the basis of molecular dynamics calculations. Recently, surface pressure area experiments have indicated that there was no evidence to suggest that the average area per molecule for ω-3 fatty acids was different from the ω-6 fatty acid at high surface pressure (19). The most interesting finding about the role of ω-3 fatty acids in plasma membranes comes from a recent work of Knapp *et al.* (44). This study demonstrates that dietary n-3 fatty acids are selectively incorporated into phospholipid species that are concentrated in the erythrocyte membrane inner leaflet. If ω-3 fatty acids in our experiments have been incorporated in the inner leaflet of the smooth muscle cells, EPA and DHA could have adopted closely packed arrays and excluded cholesterol molecules from the inner to the outer leaflet of the membrane by negative association (43). Then, cholesterol in the outer layer could be picked up by the lipoproteins and plasma membrane cholesterol efflux increased. Nevertheless, this mechanism remains to be corroborated. Further studies are needed for complete elucidation of the n-3 fatty acid metabolism in cells and particularly for *in vivo* evaluation of dietary n-3 benefits, which must be confronted with the other physiological actions of n-3, and particularly the eicosanoid mediated cellular physiology (45).

ACKNOWLEDGMENTS

E.D. was a recipient of a training research grant from Pierre Fabre Santé Laboratories. This work was supported by INSERM. The authors gratefully thank Dr. Philippe Moulin for helpful comments, Chantal Covacho for her skillful assistance in cell culture and John Carew for critically reading this manuscript.

REFERENCES

1. Rosenfeld, M.E., Tsukada, T., Chait, A., Bierman, E.L., Gown, A.M., and Ross, R. (1987) *Arteriosclerosis 7,* 24–34.
2. Rosenfeld, M.E., Tsukada, T., Gown, A.M., and Ross, R. (1987) *Arteriosclerosis 7,* 9–23.
3. Dartsch, P.C., Bauriedel, G., Schinko, I., Weiss, H.D., Hofling, B., and Betz, E. (1989) *Atherosclerosis 80,* 149–157.
4. Oram, J.F. (1990) *Curr. Opin. Lipidol. 1,* 416–421.
5. Rothblat, G.H., and Phillips, M.C. (1991) *Curr. Opin. Lipidol. 2,* 288–294.
6. Mahlberg, F.H., and Rothblat, G.H. (1992) *J. Biol. Chem. 267,* 4541–4550.
7. Spector, A.A., and Yorek, M.A. (1985) *J. Lipid. Res. 26,* 1015–1035.
8. Kinsella, J.E., Lokesh, B., and Stone, R.A. (1990) *Am. J. Clin. Nutr. 52,* 1–28.
9. Dusserre, E., Bourdillon, M.C., Ciavatti, M., Covacho, C., and Renaud, S. (1993) *Lipids 28,* 589–592.
10. Kilsdonk, E.P.C., Dorsman, A.N.R.D., Van Gent, T., and Van Tol, A. (1992) *J. Lipid. Res. 33,* 1373–1382.
11. Folch, J.M., Lees, M., and Sloane–Stanley, G.H. (1987) *J. Biol. Chem. 226,* 497–509.
12. Morrison, W.J., and Smith, M.M. (1964) *J. Lipid. Res. 5,* 600–608.
13. Kostner, G.M., Avogaro, P., Bittolo–Bon, G., Crazzoloto, G., and Quinci, G.B. (1979) *Clin. Chem. 25,* 939–942.
14. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248–254.
15. Brinton, E.A., Oram, J.F., Chen, C.H., Albers, J.J., and Bierman, E.L. (1986) *J. Biol. Chem. 261,* 495–503.
16. Rothblat, G.H., Bamberger, M., and Phillips, M.C. (1983) *Methods in Enzymology 129,* 628–644.
17. Oram, J.F., Mendez, A.J., Slotte, J.P., and Johnson, T.F. (1991) *Arteriosclerosis and Thrombosis 11,* 403–414.
18. Lange, Y. (1992) *J. Lipid. Res. 33,* 315–321.
19. Urquhart, R., Chan, R.Y.S., Li, Q–T., Tilley, L., Grieser, F., and Sawyer, W.H. (1992) *Biochem. Intern. 26,* 831–841.
20. MacFarlane, A.S. (1958) *Nature 182,* 53.
21. Omodeo Sale, F., Marchesini, S., Fishman, PH., and Berra, B. (1984) *Anal. Biochem. 142,* 374–390.
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
23. Grundy, S.M., and Denke, M.A. (1990) *J. Lipid. Res. 31,* 1149–1172.
24. Kim, D.N., Schmee, J., and Thomas, W.A. (1990) *Atherosclerosis 81,* 209–216.
25. Parks, J.S., and Rudel, L.L. (1990) *Atherosclerosis 84,* 83–94.
26. Lindsey, S., Pronczuck, A., and Hayes, K.C. (1992) *J. Lipid. Res. 33,* 647–658.
27. Murthy, S., Albright, E., Mathur, SN., and Field, F.J. (1988) *J. Lipid. Res. 29,* 773–780.
28. Sorci–Thomas, M., Hendricks, C.L., and Kearns, M.W. (1992) *J. Lipid Res. 33,* 1147–1156.
29. Kuo, P., Weinfeld, M., Rudd, M.A., Amarante, P., and Loscalzo, J. (1990) *Arteriosclerosis 10,* 111–118.
30. Rothblat, G.H., Mahlberg, F.H., Johnson, W.J., and Phillips, M.C. (1992) *J. Lipid. Res. 33,* 1091–1097.
31. Yerram, N., and Spector, A.A. (1989) *Lipids 24,* 594–602.
32. Morisaki, N., Kanzaki, T., Fujiyama, Y., Osawa, I., Shirai, K., Matsuoka, N., Saito, Y., and Yoshida, S. (1985) *J. Lipid. Res. 26,* 930–939.
33. Davis, P.J., and Evans, J. (1992) *Nutrition Res. 12,* 857–865.
34. Stillwell, W., Ehringer, W., and Jenski, L.J. (1993) *Lipids 28,* 103–108.
35. Sweet, W.D., and Schroeder, F. (1988) *FEBS Letters. 229,* 188–192.
36. Kilsdonk, E.P.C., Van Gent, T., Dorsman, A.N.R.D., and Van Tol, A. (1992) *Atherosclerosis 97,* 131–142.
37. Hokland, B., Mendez, A.J. and Oram, J.F. (1992) *J. Lipid. Res. 33,* 1335–1342.
38. Aviram, M., Bierman, E.L., and Oram, J.F. (1989) *J. Lipid. Res. 30,* 65–76.
39. Esteva, O., Baudet, M.F., Lasserre, M., and Jacotot, B. (1986) *Biochim. Biophys. Acta. 875,* 174–182.
40. Goodnight, S.H. (1991) *Trends Cardiovasc. Med. 1,* 112–116.
41. Schroeder, F., Jefferson, J.R., Kier, A.B., Knittel, J., Scallen, T.J., Wood, W.G., and Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med. 196,* 235–252.
42. Schroeder, F., Kier, A.B., and Sweet, W.D. (1990) *Arch. Biochem. Biophys. 276,* 55–64.
43. Applegate, K.R., and Glomset, J.A. (1986) *J. Lipid Res. 27,* 658–680.
44. Knapp, H.R., Hullin, F., and Salem, N., Jr. (1994) *J. Lipid Res. 35,* 1283–1291.
45. Lands, W.E.M. (1992) *FASEB J. 6,* 2530–2536.

[Received August 10, 1994, and in revised form November 4, 1994; Revision accepted December 8, 1994]

Studies on Linoleic Acid 8*R*-Dioxygenase and Hydroperoxide Isomerase of the Fungus *Gaeumannomyces graminis*

Chao Su, Irene D. Brodowsky* and Ernst H. Oliw

Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University
Biomedical Center, S-751 24 Uppsala, Sweden

ABSTRACT: Linoleic acid is sequentially converted to 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid by the 8*R*-dioxygenase and hydroperoxide isomerase of the fungus *Gaeumannomyces graminis*, which is a common pathogen of wheat. The objective of this study was to separate and characterize the two enzyme activities. The isomerase activity was found mainly in the microsomal fraction of the mycelia and the 8*R*-dioxygenase in the cytosol. The 8*R*-dioxygenase could be partially purified by ammonium sulfate precipitation, gel filtration, ion exchange chromatography or isoelectric focusing. The 8*R*-dioxygenase was unstable during purification, but it could be stabilized by glutathione, glutathione peroxidase and ethylenediaminetetraacetic acid. Several protease inhibitors reduced the enzyme activity. Gel filtration with Sephacryl S-300 showed that most 8*R*-dioxygenase activity was eluted with the front with little retention. Isoelectric focusing in the presence of ethylene glycol (20%) indicated an isoelectric point of pI 6.1–6.3. The enzyme was retained on strong anion exchange columns at pH 7.4 and could be eluted with 0.3–0.5 M NaCl. Incubation of the enzyme with 0.1 mM linoleic acid led to partial inactivation, which may indicate product inhibition. Paracetamol and the lipoxygenase inhibitor ICI 230,487 at 30 μM inhibited the 8*R*-dioxygenase by 44 and 58%, respectively. 8*R*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid was isolated from incubations of linoleic acid with the partially purified enzyme or with the cytosol in the presence of *p*-hydroxymercuribenzoate. The hydroperoxide was rapidly converted by the hydroperoxide isomerase in the microsomal fractions to 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid. The isomerase was neither inhibited by carbon monoxide nor by ketoconazole (100 μM). The isomerase was active over a broad pH range. It could be separated from the 8*R*-dioxygenase by differential centrifugation, by ammonium sulfate precipitation and by gel filtration. We conclude that the linoleic acid 8*R*-dioxygenase and the hydroperoxide isomerase are two separate enzymes.

Lipids 30, 43–50 (1995).

Lipoxygenases metabolize linoleic acid and other polyunsaturated fatty acids with a 1*Z*,4*Z*-pentadienyl functionality to hydroperoxy fatty acids by hydrogen abstraction from the methylene carbon and antarafacial insertion of molecular oxygen (1,2). With few exceptions, the known sequences of lipoxygenases of plants and mammals show a common central motif, His-X_4-His-X_4-His-X_{17}-His-X_8-His, with at least five other conserved residues in this region (3,4). This central area is believed to bind the catalytically active iron. The three-dimensional structure of soybean lipoxygenase-1 and its iron-binding amino acid residues were recently reported (5,6). The catalytic iron is bound to three histidines and the carboxyl group of the C-terminal isoleucine. The iron is the target of many lipoxygenase inhibitors (7).

Two fungi, *Gaeumannomyces graminis* and *Laetisaria arvalis*, have been found to contain a fatty acid dioxygenase that shows both catalytic similarities and differences with lipoxygenases (8–10). This enzyme, linoleic acid 8*R*-dioxygenase, transforms linoleic acid to 8*R*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid (8*R*-HPODE) by hydrogen abstraction and antarafacial insertion of molecular oxygen at C-8 (11). In contrast to lipoxygenases, the 8*R*-dioxygenase accepts oleic acid as a substrate. It can metabolize many C_{18} fatty acids with a saturated C-2 to C-8 carboxyl side chain and with a *cis* double bond at position 9–10 (9). Linoleic acid 8*R*-dioxygenase and lipoxygenases are both inhibited by hydroxamic acid derivatives and by some other agents, indicating a similar oxygenation mechanism (12).

Hydroperoxides of fatty acids can be further metabolized. *Gaeumannomyces graminis* has been found to metabolize linoleic acid to 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic

*To whom correspondence should be addressed at Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University BMC, P.O. Box 591, S-751 24 Uppsala, Sweden.

Abbreviations: 7*S*,8*S*-DiHODE, 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylene-*bis*(oxyethylenenitrilo)]tetraacetic acid; GC/MS, gas chromatography/mass spectrometry; GSH, glutathione; 8*R*-HODE, 8*R*-hydroxy-9*Z*,12*Z*-octadecadienoic acid; 13*S*-HODE, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; 9*S*-HPODE, 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid; 8*R*-HPODE, 8*R*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid; P450, cytochrome P450; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; UV, ultraviolet.

Copyright © 1995 by AOCS Press

acid (7*S*,8*S*-DiHODE) (9). Experiments under oxygen-18 gas and with glutathione peroxidase indicated that 7*S*,8*S*-DiHODE was formed from 8*R*-HPODE by an intramolecular hydroxylation by the distal hydroperoxide oxygen (9). The hydroxylation at C-7 of 8*R*-HPODE, which is catalyzed by this hydroperoxide isomerase, occurs by suprafacial hydrogen abstraction and oxygen insertion, resulting in a *threo* 1,2-diol (12). A similar hydroperoxide isomerase has also been described in the red alga, *Gracilariopsis lemaneiformis*, which can metabolize hydroperoxides to *erythro* 1,2-diols, e.g., 12*S*-hydroperoxyeicosatetraenoic acid to 12*R*,13*S*-dihydroxyeicosatetraenoic acid (13,14). Another example of a hydroperoxide metabolizing enzyme is the allene oxide synthase from flaxseed. This enzyme was recently cloned, and it belongs to a new gene family of cytochrome P450 (P450) (CYP74) (15,16).

The objectives of the present study were to determine whether the 8*R*-dioxygenase and hydroperoxide isomerase activities could be separated and to study some properties of the two enzymes. We describe methods to prepare linoleic acid 8*R*-dioxygenase, hydroperoxide isomerase and 8*R*-HPODE. Measures to increase the stability of the 8*R*-dioxygenase are also described.

MATERIALS AND METHODS

Materials. Gaeumannomyces graminis var. graminis was from Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). Linoleic acid (99%), ketoconazole, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *p*-hydroxymercuribenzoate, superoxide dismutase and glutathione (GSH) peroxidase (both from bovine erythrocytes) were from Sigma (St. Louis, MO). [1-^{14}C]Linoleic, [1-^{14}C]arachidonic and [1-^{14}C]eicosapentaenoic acids (55 Ci/mol) were purchased from Amersham Int. (Amersham, Buckshire, United Kingdom). Fatty acids were checked by thin-layer chromatography (TLC) and purified if necessary. SepPak-C_{18} was from Waters (Milford, MA). Precoated TLC plates (0.25 mm silica gel 60A, 10 or 20 cm long) were from Merck (Darmstadt, Germany) and from Whatman (Maidstone, United Kingdom). 8*R*-HODE, 7*S*,8*S*-DiHODE, 18*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid and 19*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid were prepared as described (8,9,17). 13*S*-Hydroxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HODE) was obtained by biosynthesis and used as internal standard (12). 9*S*-Hydroperoxy-10*E*-12*Z*-octadecadienoic acid (9*S*-HPODE) was prepared as described (18). Sephacryl S-300 HR, Sephadex G-50, PD-10 and Q-Sepharose FF were from Pharmacia (Uppsala, Sweden). Ampholyte (3:10 and 5:8) and strong anion exchange columns (Econopak Q) were from BioRad (Stockholm, Sweden). Paracetamol was obtained locally. ICI 230,487, which is the N-ethyl analogue of the 5-lipoxygenase inhibitor ICI D2138 (*cf.* Ref. 7), was a gift from Zeneca Pharmaceuticals (Cheshire, United Kingdom). BW A4C (7) was a gift from the Wellcome Foundation (Beckenham, United Kingdom). Aprotinin was from Bayer AG (Leverkusen, Germany). Emulgen 911 was a generous gift from KAO Corp. (Tokyo, Japan). 4-Aminobenzamidine dihydrochloride and most other chemicals were from Merck.

Preparation of subcellular fractions of mycelia. Gaeumannomyces graminis was cultivated for about one week as described (9). The mycelia were filtered under vacuum, washed with saline and stored at –80°C. The following steps were performed at 4°C. The frozen mycelia (10 g) were homogenized with alumina type A-5 (10 g; Sigma) and 20 mL 50 mM potassium phosphate buffer (pH 7.4; buffer A) with 0.75 M sorbitol, by grinding in a mortar on ice. Addition of GSH (2 mM), ethylenediaminetetraacetic acid (EDTA; 5 mM) and [ethylene-*bis*(oxyethylenenitrilo)]tetraacetic acid (EGTA; 1–2 mM) to the buffer was found to improve enzyme stability. The white slurry was centrifuged at 10,000 × *g* for 20 min. The supernatant was saved, and the alumina pellet was ground in 10–15 mL buffer and recentrifuged. The clear supernatants were pooled and subjected to ultracentrifugation (100,000 × *g*; 60 min). The 100,000 × *g* supernatant and the pellet, which was suspended in buffer A, are referred to as the cytosol and the microsomal fraction, respectively. The cytosol and the microsomes were either used directly or stored at –80°C.

Assay of linoleic acid 8R-dioxygenase. All incubations were performed on ice. The cytosol was diluted to a protein concentration of about 0.5 mg/mL (with buffer A or 10 mM triethanolamine, pH 7.4) and incubated with 2 to 100 μM [^{14}C]18:2(n-6). An enzyme unit of 8*R*-dioxygenase was defined as biosynthesis of 1 pmol of 8*R*-HODE per min upon incubation with 100 μM [^{14}C]linoleic acid (3–4 Ci/mol), GSH-peroxidase (1 U/mL) and GSH (1 mM) for 10 min [reverse-phase high-performance liquid chromatography (RP–HPLC) assay]. This concentration of GSH–peroxidase inhibited formation of 7*S*,8*S*-DiHODE (9). The cytosol typically contained 400–1000 units per mg of protein. The 8*R*-dioxygenase activity was followed during enzyme purification by incubation of 0.2–0.3 mL of the fractions with 60 nCi of [^{14}C]linoleic acid (20 min, TLC assay). All incubations were terminated by addition of four volumes of ethanol, and precipitates were removed by centrifugation. The material in the supernatant was extracted and analyzed by RP–HPLC or TLC.

Drugs were added to the cytosol (with GSH and GSH-peroxidase) and preincubated on ice for 20 min. The substrate was then added (0.1 μCi of [^{14}C]linoleic acid, 2 μM). The drugs were dissolved in ethanol (ICI 230,487), dimethyl sulfoxide (BW A4C) or distilled water (paracetamol), and these solvents (0.6% final concentration) were added to the controls. Samples were incubated in duplicate and analyzed by RP–HPLC. The effects of detergents, protease inhibitors and solvents on 8*R*-dioxygenase activity were assayed by TLC.

Assay of hydroperoxide isomerase. The microsomes were suspended in buffer A (to 2 mg protein/mL) and incubated with ^{14}C-labeled 8*R*-HPODE (3–5 μCi) in duplicate or triplicate (20 min, on ice). All incubations were terminated by the addition of 4 vol of ethanol. The effect on variation of buffer

pH on isomerase activity was assessed by adding 5 μL of the microsomal suspension to 95 μL 0.1 M buffer of different pH values. The buffers were citrate–phosphate (pH 3–7), potassium phosphate (pH 6–8), sodium borate (pH 8–9) in one series of experiments and glycine–NaOH (pH 9–12) in a second experiment. The buffered enzyme was incubated with [^{14}C]8*R*-HPODE for 10 min. In order to approximate the useful incubation times for the isomerase at our experimental conditions, the microsomal suspension was incubated with [^{14}C]8*R*-HPODE, and duplicate samples were removed at different time points. To determine if ketoconazole inhibited the isomerase, the microsomal suspension was pretreated for 20 min with 100 μM ketoconazole (in 0.07 M HCl). To establish whether CO inhibits the isomerase, buffer A (7 vol) on ice was saturated with CO or N_2, mixed with the microsomes (1 vol) and incubated directly. Formation of [^{14}C]7*S*,8*S*-DiHODE was determined by RP–HPLC and liquid scintillation, using 13*S*-HODE as an internal standard.

Separation of metabolites. After extractive isolation with SepPak-C_{18} cartridges (8), the metabolites formed from [^{14}C]linoleic acid were analyzed by RP–HPLC using 13*S*-HODE (1 μg) as internal standard or by TLC. The RP–HPLC columns (5-μm Nucleosil-C_{18}, 150 × 4.6 mm) were eluted with methanol/water/acetic acid (75:25:0.01, by vol) at 1 mL/min. Ultraviolet (UV) absorption at 235 nm was followed for quantification of the internal standard (8). Equipment for RP–HPLC was as described (8). TLC was usually performed with toluene/dioxane/acetic acid/formic acid (82:14:1:1; by vol; system A). The R_f values for linoleic acid, 8*R*-HPODE, 8*R*-HODE and 7*S*,8*S*-DiHODE were 0.54, 0.42, 0.32 and 0.13, respectively. For quantification, the TLC plates were scanned for radioactivity (model II; Berthold Dünnschichtscanner, Hässleby, Sweden). The area of the ^{14}C-labeled peaks on the chart was usually estimated by weighing the peaks. Initially, authentic 8*R*-HODE and 7*S*,8*S*-DiHODE were used as TLC references and stained with phosphomolybdic acid (Merck). Methyl esters were prepared by treatment with an excess of ethereal diazomethane (9). 7*S*,8*S*-DiHODE methyl ester was analyzed by TLC (ethyl acetate/hexane/acetic acid, 5:5:0.05; by vol). The R_f values for 7*S*,8*S*-DiHODE methyl ester and 8*R*-HODE methyl ester were 0.46 and 0.66, respectively.

*Preparation of ^{14}C-labeled 8*R*-HPODE.* A partially purified preparation of 8*R*-dioxygenase was used to biosynthesize 8*R*-HPODE. The low-speed supernatant was desalted (by gel filtration on Sephadex G-50 or PD-10 columns in buffer A with 0.75 M sorbitol), and the active fractions were subject to anion exchange chromatography (three Econopac Q cartridges in a series). The latter were eluted with a linear gradient of 0–1 M NaCl in 30 min (flow rate 1 mL/min) in buffer A. The active fractions (0.3 to 0.5 M NaCl) were incubated with [^{14}C]linoleic acid (10 μCi) on ice.

Alternatively, 18 mL of buffer A with 0.75 M sorbitol was bubbled with oxygen gas for 5 min on ice. [^{14}C]Linoleic acid (7 μCi in 50 μL ethanol) was added together with 0.2 mL 100 μM *p*-hydroxymercuribenzoate, followed by 2 mL of cytosol (containing 40 mg of protein and 28,000 units of 8*R*-dioxygenase). After mixing, the sample was incubated for 15 min under a stream of oxygen gas. After acidification and extractive isolation, the products were purified by TLC (system A). The band with [^{14}C]8*R*-HPODE was scraped from the TLC plate and eluted with ethyl acetate.

Partial purification of 8R-dioxygenase. Ammonium sulfate precipitates were prepared by mixing equal volumes of cytosol and a 3.6 M (90% saturation) ammonium sulfate solution in buffer A (pH 7.4), containing 2 mM GSH and 5 mM EDTA. After mixing, the suspension was centrifuged (10,000 × *g*, 20 min) and the pellet was either resuspended in phosphate buffer with 2 mM GSH and 5 mM EDTA or stored at –80°C.

Sephacryl S-300 (1.9 × 49 cm) was used for gel filtration. Protein elution was followed by UV absorption at 280 nm. The column was usually eluted with 40 mM potassium phosphate buffer (pH 7.4, flow 2.2 mL/min) containing 5 mM EDTA and 2 mM GSH (buffer B). In some experiments, ethylene glycol (20%) or sorbitol (14%) were added. A more diluted buffer was used (5 mM potassium phosphate) in connection with isoelectric focusing.

The active fractions from gel filtration were diluted with water and subjected to isoelectric focusing with 1% ampholyte (3:10 or 5:8) and 20% ethylene glycol. A preparative scale isoelectric focusing cell (Rotofor; BioRad) was used. Ethylene glycol reduced precipitations in the Rotofor cell and allowed electrofocusing at a low temperature. The power was increased stepwise: 3W (0.5 h), 9W (0.5 h) and 12W (2 h). Refocusing was performed with fractions of pH 4–9. The 20 fractions obtained were adjusted to pH 7.0–7.5 with buffer, and enzyme activity was analyzed by TLC assessment of products.

Ion exchange chromatography was performed with strong anion exchangers, Econopac Q cartridges (see above) or a Q-Sepharose FF column (1.6 × 7 cm). The latter was equilibrated with buffer B and eluted with 3 mL/min. The column was typically loaded with 20 mg protein, washed with buffer B for 15 min and then eluted with a concave gradient of 0–1 M NaCl in buffer B.

Assay of cytochrome P450 metabolites. The microsomes and cytosol were incubated with [^{14}C]arachidonic acid or [^{14}C]eicosapentaenoic acid (1.2 μCi/mL) for 30 min at 30°C in the presence of 1 mM NADPH. After extractive isolation, the metabolites of arachidonic acid and eicosapentaenoic acid were analyzed by RP–HPLC or methylated and analyzed by TLC (8,9). Sonication of microsomes was performed as described (8).

Other analyses. Gas chromatography/mass spectrometry (GC/MS) analysis was performed as described (9). Radioactivity was determined by liquid scintillation using EcoScint (Hintze, Lidingö, Sweden). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the manufacturer of electrophoresis equipment (Miniprotean II; BioRad). Protein concentrations were determined as described (19) with gamma-globulin as standard or by UV analysis (280 nm).

RESULTS

*Stability and partial purification of linoleic acid 8*R*-dioxygenase.* Purification of 8*R*-dioxygenase was hampered by the instability of the enzyme. Storage for 24 h at 4°C usually led to loss of all or almost all enzyme activity. Addition of mM concentrations of GSH stabilized the enzyme. In many experiments, half of the activity of the cytosol could be preserved for 3 d in the presence of 2 mM GSH, while the control showed no enzyme activity after this time. Addition of glutathione peroxidase (2 U/mL) further increased the stability for a few days (Fig. 1). Superoxide dismutase (2 mg/mL) or cysteine (1 mM) appeared to be without effect. ATP (2 mM) and calcium (about 2 mM free concentration) did not stimulate the enzyme.

Some protease inhibitors were evaluated. EDTA (5 mM) stabilized the enzyme in comparison with the control. However, 1 mM TCPK or 1 mM 4-aminobenzamidine inhibited the 8*R*-dioxygenase activity by over 70%, and aprotinin (100 U/mL) was without beneficial effects.

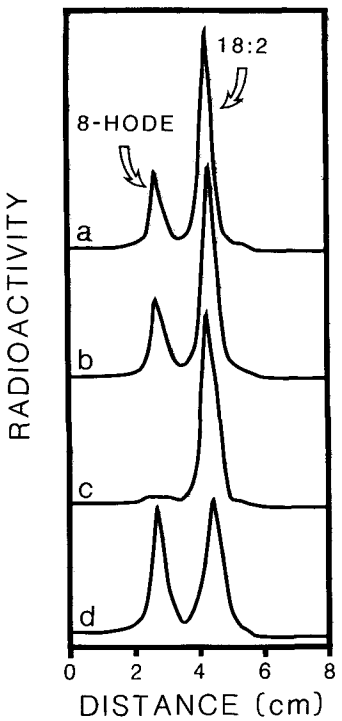


FIG. 1. Effect of glutathione peroxidase on the stability of linoleic acid 8*R*-dioxygenase. The cytosolic fraction of *Gaeumannomyces graminis* was kept on ice for 7 d (a–c) or incubated on the first day with [^{14}C]linoleic acid (d). The products were analyzed by thin-layer chromatography (system A; toluene/dioxane/acetic acid/formic acid, 82:14:1:1; by vol). Trace a: analysis of products from proteins stored for 7 d in the presence of glutathione (GSH) peroxidase (2 U/mL) and superoxide dismutase (2 mg/mL); trace b: analysis of products from proteins stored for 7 d with GSH peroxidase; trace c: analysis of products from proteins stored for 7 d without additions; d: analysis of products from proteins incubated on day 1 without GSH peroxidase and superoxide dismutase. Ethyldiaminetetraacetic acid, 5 mM, and GSH, 3 mM , were present in all four samples. This preparation apparently did not contain hydroperoxide isomerase activity.

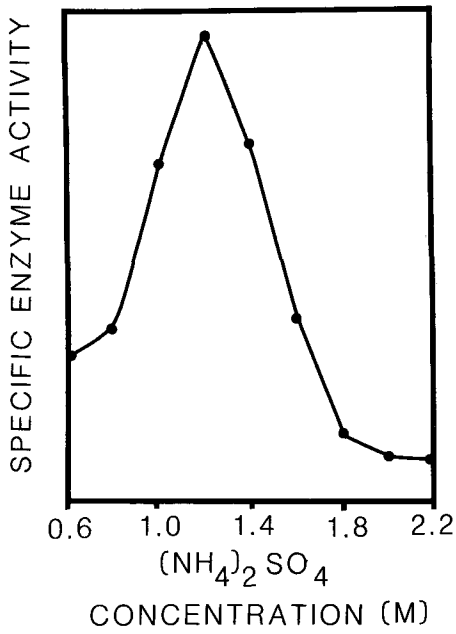


FIG. 2. Ammonium sulfate precipitation of linoleic acid 8*R*-dioxygenase in the cytosol of *Gaeumannomyces graminis*. The curve shows specific enzyme activity in the precipitates, taken from zero to the indicated concentration of ammonium sulfate.

Sorbitol (0.75M, 14%), glycerol (15%) and ethylene glycol (25%) did not inhibit the enzyme. Sorbitol appeared to increase the yield of 8*R*-dioxygenase during grinding of mycelia. Detergents and organic solvents, such as 0.5% of Triton X-100, Emulgen 911, Tween 80 and 20, sodium deoxycholate or polyoxyethylene-10-lauryl ether, 10% isopropanol, and 10% acetonitrile, inactivated the enzyme. Urea (1–4 M) and glycine (2%) also inhibited the enzyme, while ampholyte (1%) was without effect.

The precipitation of 8*R*-dioxygenase from the cytosol by increasing concentrations of ammonium sulfate is illustrated in Figure 2. Most enzyme activity was precipitated between 20 and 45% saturation, i.e., between 0.8 and 1.8 M ammonium sulfate. Routinely, the 0 to 45% ammonium sulfate cut was used for further purification. The recovery of enzyme activity was 75%, and the specific activity was 0.5 nmol/min/mg of protein before and 0.6 nmol/min/mg of protein after precipitation.

Gel filtration of the ammonium sulfate precipitate showed that most of the enzyme activity eluted with the front with only little retention relative to the bulk of proteins while the isomerase activity appeared to elute with the front and with no retention relative to the bulk proteins (Fig. 3). Virtually all enzyme activity eluted before bovine serum albumin. SDS–PAGE showed that the active fractions contained proteins of various sizes. Gel filtration was therefore performed under different conditions to reduce formation of aggregates of proteins, e.g., with 20% ethylene glycol (20). However, the elution of proteins and enzyme activity appeared to be un-

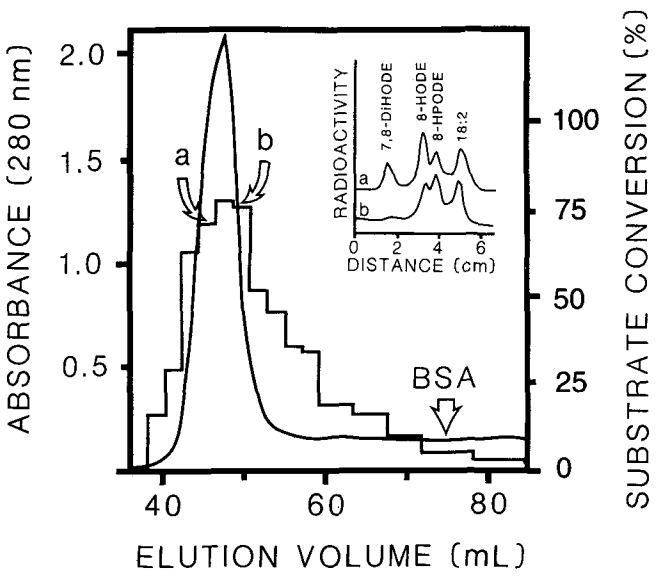


FIG. 3. Gel filtration of 8*R*-dioxygenase. The ordinates show the elution of protein (ultraviolet 280 nm; smooth curve) and 8*R*-dioxygenase activity from the column (Sephacryl-300; Pharmacia, Uppsala, Sweden). The enzyme activity of the fractions is given as the conversion of [^{14}C]linoleic acid to polar products in percent [7*S*,8*S*-DiHODE and 8*R*-H(P)ODE; thin-layer chromatographic (TLC) analysis]. The elution volume of bovine serum albumin (BSA) is marked with the arrow. The inserted TLC chromatograms of fractions marked a and b indicate a partial separation of the 8*R*-dioxygenase from the isomerase.

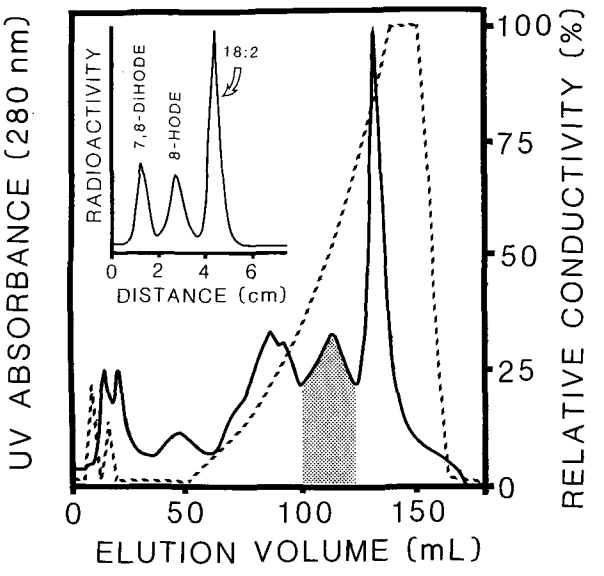


FIG. 4. Chromatography of linoleic acid 8*R*-dioxygenase on a strong anion exchange column. The column (Q-Sepharose FF) was eluted with a gradient of 0–1 M NaCl as shown by the dotted line (relative conductivity). Elution of proteins and other ultraviolet (UV)-absorbing material is shown by the solid line. Linoleic acid 8*R*-dioxygenase activity eluted with 100–125 mL (shaded area). The inserted TLC analysis shows that these fractions were associated with hydroperoxide isomerase activity, at least in some experiments. Abbreviations as in Figure 3.

changed. After gel filtration, the specific activity was not increased (0.5 nmol/min/mg of protein).

Most of the enzyme activity was retained on strong anion exchange columns (Econopac Q, Q-Sepharose FF) when dissolved ammonium sulfate precipitate was applied. The bound enzyme could be eluted with a gradient of 0–1 M NaCl. A chromatogram from a column with Q-Sepharose FF is illustrated in Figure 4. As shown by the insert in Figure 4, the fractions with 8*R*-dioxygenase in some experiments also contained hydroperoxide isomerase. The isomerase activity was not analyzed *per se*, but formation of [^{14}C]7*S*,8*S*-DiHODE from [^{14}C]linoleic acid or [^{14}C]8*R*-HPODE indicated its presence. Anion exchange chromatography was also associated with significant loss of enzyme activity. The specific activity was not determined.

Isoelectric focusing of active fractions from gel filtration was performed in the presence of 20% ethylene glycol. About 5 mg of protein could be loaded without significant protein precipitation. The apparent isoelectric point of 8*R*-dioxygenase was about pI 6.1 to 6.3, as judged from refocusing. The active fractions from the isoelectric focusing appeared to be without hydroperoxide isomerase activity. Other fractions were not checked for isomerase activity. SDS–PAGE showed that the active fractions from isoelectric focusing were not homogeneous. Isoelectric focusing led to a major loss of enzyme activity. The specific activity was not determined.

Inhibition of linoleic acid 8R-dioxygenase by linoleic acid and by drugs. Many lipoxygenases are inactivated by their products (21). We investigated whether 8*R*-dioxygenase exhibited this phenomenon. Unlabeled linoleic acid (0.1 mM) was first added to the cytosol (0.3 mL) and incubated for 1 h. Radiolabeled linoleic acid (0.1 μCi) was then added, and the samples were further incubated for 1 h. The controls were preincubated without substrate for 1 h, and the two substrates were then added and incubated for 1 h.

Preincubation of the cytosol with 0.1 mM linoleic acid for 1 h on ice led to loss of enzyme activity. The conversion of the radiolabeled substrate with the preincubated cytosol was 53 ± 4% (SD, n = 4) vs. 100 ± 5% in the control (n = 4). In the presence of 1 mM GSH, the corresponding figures were 48 ± 7% (n = 6) vs. 100 ± 6% (n = 6) in the control. Preincubation with linoleic acid thus reduced the enzyme activity to half, and GSH had no stabilizing effect during this short time period.

Preliminary experiments showed that 8*R*-dioxygenase was inhibited dose-dependently by paracetamol and by ICI 230,487. In the presence of 30 μM of paracetamol the 8*R*-dioxygenase activity was 56 ± 9% (SD, n = 7). In the presence of 30 μM of ICI 230,487 the dioxygenase activity was 42 ± 7% (n = 5) of the controls (100 ± 7%, n = 11). For comparison, another lipoxygenase inhibitor, BW A4C, caused over 90% inhibition of this preparation at 3 μM (*cf.* Ref. 12).

Preparation of 8R-HPODE. Incubation of the cytosol with linoleic acid usually led to formation of 8*R*-HODE as the

main product rather than 8*R*-HPODE. In the presence of 1 mM *p*-hydroxymercuribenzoate, the yield of 8*R*-HPODE increased. Under these conditions, ^{14}C-labeled linoleic acid was converted to ^{14}C-labeled 8*R*-HPODE as the major product as shown on the TLC chromatogram in Figure 5A. Alternatively, 8*R*-HPODE was prepared by incubation of linoleic with 8*R*-dioxygenase that had been partly purified by anion exchange chromatography. In the latter case, TLC afforded 18:2, 8*R*-HPODE and 8*R*-HODE in a ratio of 1:2:1.

Properties of hydroperoxide isomerase. The microsomal fraction of the mycelia contained relatively little 8*R*-dioxygenase, but a prominent hydroperoxide isomerase activity. Incubation of the microsomal fraction with [^{14}C]linoleic acid led to conversion of the substrate to one major, polar metabolite with little accumulation of 8*R*-H(P)ODE. The structure of this metabolite was confirmed by GC/MS to be 7*S*,8*S*-DiHODE [analyzed as the $(CH_3)_3Si$ ether methyl ester derivative]. The C value was 20.9, and the mass spectrum was identical with that previously reported for 7*S*,8*S*-DiHODE (9). Incubation of [^{14}C]8*R*-HPODE with the microsomal fraction resulted in biosynthesis of [^{14}C]7*S*,8*S*-DiHODE as the major product as judged from RP–HPLC (Fig. 5B). No conversion was noted by heat-inactivated preparations (2 min in boiling water). Hydroperoxide isomerase activity was also found in some preparations of cytosol as previously reported (9,12). Neither CO nor ketoconazole (100 μM) inhibited the microsomal metabolism of [^{14}C]8*R*-HPODE to [^{14}C]7*S*,8*S*-DiHODE.

As shown by Figure 5C, [^{14}C]8*R*-HPODE was rapidly converted to [^{14}C]7*S*,8*S*-DiHODE. The effect of pH on the isomerase activity is shown by the insert in Figure 5C. The isomerase appeared to have a broad pH optimum with the enzyme being active between pH 7 and 10.

The methyl ester of [^{14}C]8*R*-HPODE was also investigated as a substrate. TLC showed that the methyl ester was partly converted to [^{14}C]7*S*,8*S*-DiHODE methyl ester (product and substrate in a ratio of about 1:3), while the free acid was converted more efficiently by the microsomes under the same conditions [7*S*,8*S*-DiHODE and 8*R*-H(P)ODE in a 2:1 ratio]. These results suggested that the methyl ester of 8*R*-HPODE was a rather poor substrate for the isomerase. Finally, [^{14}C]9*S*-HPODE was not metabolized to diols by the microsomes.

P450 in microsomal and cytosolic fractions. Both the cytosolic and the microsomal fractions metabolized radiolabeled arachidonic acid and eicosapentaenoic acid in the presence of 1 mM NADPH to polar products. Only small amounts of products were formed without this cofactor. TLC analysis indicated that arachidonic acid was metabolized to 18*R*- and 19*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acids in a 2:3 ratio. Eicosapentaenoic acid was mainly metabolized to a more polar product than these hydroxy fatty acids (presumably 17,18-dihydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, *cf.* Refs. 8,17). The P450-catalyzed oxygenation of arachidonic acid was about eight times greater per mg of protein in the microsomal fraction than in the cytosol. However, sonication of washed microsomes and recentrifugation appeared to increase the enzyme activity in the supernatant about four times.

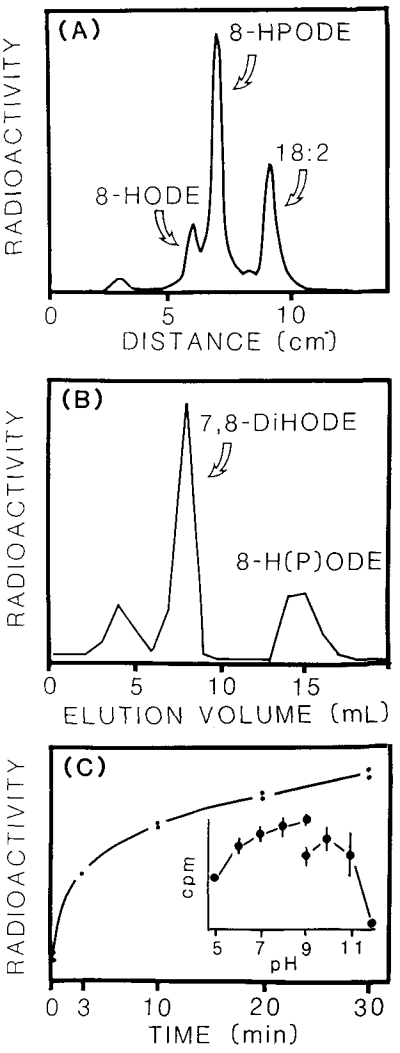


FIG. 5. Isolation and metabolism of 8*R*-HPODE. A, TLC analysis of an incubation of cytosol (10 mg protein) with [^{14}C]linoleic acid (7 μCi, 37 μg) and 1 mM *p*-hydroxymercuribenzoate (see text for details). The main peak of material corresponded to [^{14}C]8*R*-HPODE. B, reversed-phase high-performance liquid chromatography analysis of products from an incubation of [^{14}C]8*R*-HPODE (2 nCi, 10 ng) with microsomes of mycelia of *Gaeumannomyces graminis* (0.2 mg protein). C. Time curve for the formation of [^{14}C]7*S*,8*S*-DiHODE from [^{14}C]8*R*-HPODE (2 nCi, 10 ng) by microsomes of mycelia (0.2 mg protein). The insert shows the effect of pH on the conversion of [^{14}C]8*R*-HPODE to [^{14}C]7*S*,8*S*-DiHODE. Abbreviations as in Figure 3.

DISCUSSION

The present report shows that the linoleic acid 8*R*-dioxygenase and hydroperoxide isomerase activities can be separated by differential centrifugation, by ammonium sulfate precipitation and by gel filtration chromatography. The 8*R*-dioxygenase is present mainly in the cytosol and the hydroperoxide isomerase in the microsomal fractions. The isomerase is more sensitive to salting out by ammonium sulfate than the 8*R*-

dioxygenase, and the activities can be partially separated by this method. Furthermore, the 8*R*-dioxygenase activity is retained somewhat on a gel filtration column relative to the isomerase activity and the bulk proteins.

The purification of 8*R*-dioxygenase was not straightforward due to its instability. Many lipoxygenases share this feature (22,23). The reason for loss of enzyme activity is unknown, but proteolysis and loss of the catalytic iron after exposure to oxygen in the absence of reducing agents have been implicated. Some lipoxygenases are therefore purified under anaerobic conditions. We tried to purify 8*R*-dioxygenase under partly anaerobic conditions in preliminary experiments, but the yield did not improve. Many lipoxygenases (21) can be inactivated by their products, possibly *via* formation of peroxyl radicals. The latter can be reduced to inert alcohols by GSH, other reducing agents and glutathione peroxidase. Incubation of the 8*R*-dioxygenase with 0.1 mM linoleic acid also resulted in loss of enzyme activity (Fig. 5). We found that GSH improved the stability of 8*R*-dioxygenase significantly, while two other reducing agents, dithiothreitol and β-mercaptoethanol, were previously found to inhibit the enzyme (12). Glutathione peroxidase also partly stabilized the cytosolic linoleic acid 8*R*-dioxygenase, while superoxide dismutase had little beneficial effect (Fig. 1). It may be added that recombinant 5-lipoxygenase was found to be stabilized in the presence of glutathione peroxidase and superoxide dismutase (24).

8*R*-Dioxygenase could be partially purified by ultracentrifugation, ammonium sulfate precipitation, gel filtration, ion exchange chromatography and isoelectric focusing. The recovery after ammonium sulfate precipitation was reasonable (75%), but the specific activity was almost unchanged. During gel filtration, the main enzyme activity eluted with the front and with only some retention relative to the void volume. Formation of protein aggregates may occur during gel filtration. This step was therefore performed in the presence of 20% ethylene glycol (20). However, SDS–PAGE and gel filtration in the presence of 4 M urea showed that aggregates of proteins nevertheless were present. Since it cannot be excluded that the 8*R*-dioxygenase eluted in aggregates, the molecular weight could not be estimated from gel filtration.

Preparative-scale isoelectric focusing was described as a very efficient step for purification of rabbit reticulocyte 15-lipoxygenase (22). In our hands, the method was complicated by protein precipitation even with the addition of 20% ethylene glycol and could only be used for analytical purposes. Under the conditions used, the 8*R*-dioxygenase showed an isoelectric point between pI 6.1–6.3.

Isoelectric focusing as well as anion exchange chromatography were associated with loss of enzyme activity. It seemed possible that proteolysis may explain the rather low yield of 8*R*-dioxygenase during these purification steps. Unfortunately, linoleic acid 8*R*-dioxygenase was found to be inhibited by common serine protease inhibitors, e.g., by mM concentrations of 4-aminobenzamidine and TCPK. Phenylmethanesulfonyl fluoride also inhibits 8*R*-dioxygenase (12). EDTA was the only protease inhibitor which enhanced the stability of the dioxygenase. Stabilizing agents in addition to EDTA, GSH and GSH peroxidase should be helpful for further purification.

8*R*-Dioxygenase was recently found to be inhibited by some 5-lipoxygenase inhibitors. In this report, we investigated the effects of paracetamol and ICI 230,487, a hydroxamic acid derivative, on the 8*R*-dioxygenase. Both drugs appeared to be equally potent with an IC_{50} of about 30 μM. The effect of paracetamol appeared to be dose-dependent and occurred within the clinical concentration (about 60 μM; Ref. 25). The inhibition caused by paracetamol is of interest since the mechanism of its analgesic actions is largely unknown (25). It may therefore be of interest to determine whether 8*R*-dioxygenase-like enzymes occur in mammals.

Many P450 enzymes catalyze hydroxylations with suprafacial hydrogen abstraction and oxygen insertion and can use hydroperoxides as oxygen donors (11,26,27). The microsomal hydroperoxide isomerase converted radiolabeled 8*R*-HPODE to 7*S*,8*S*-DiHODE. Like P450, it showed a suprafacial relationship of hydrogen abstraction and oxygen insertion which may indicate that the isomerase could be related to P450 (11). On the other hand, it was neither inhibited by carbon monoxide nor by ketoconazole. The microsomal fraction also contained a putative P450, which catalyzed ω2 and ω3 hydroxylation of arachidonic acid and ω3 epoxygenation of eicosapentaenoic acid. This enzyme was previously found in the cytosol and was NADPH-dependent (8).

The physiological functions of 8*R*-HODE, 8*R*-HPODE and 7*S*,8*S*-DiHODE are unknown, but it is possible that these oxylipins may have biological activity. Racemic 8*R*-HODE has been found to induce hyphal lysis of some fungi (28). In pursuit of a signal substance that induced sexual sporulation, 8*R*-HODE was detected in a mutant of the fungus *Aspergillus nidulans* and found to induce premature sexual sporulation (29,30). Interestingly, 5*S*,8*R*-dihydroxylinoleic acid was also identified as a metabolite of *A. nidulans* with a similar biological effect. It will be interesting to determine whether this compound is derived from 8*R*-HPODE.

Recently, a novel linoleic acid ω6-lipoxygenase of infected rice was sequenced by methods based on the sequence homology of lipoxygenases (31). Sequences of at least 14 different plant and mammalian lipoxygenases thus display, *inter alia,* a characteristic motif of 38 amino acids, including four conserved histidines within and one histidine outside this motif (3,4,32). If linoleic acid 8*R*-dioxygenase contains this homologous region, it is possible that the enzyme might be cloned and sequenced in analogy with the rice lipoxygenase. Otherwise, the purification of 8*R*-dioxygenase must be pursued in order to obtain a pure enzyme, which can be used as a starting point for cloning methods. *Gaeumannomyces graminis* grows rapidly and contains large amounts of 8*R*-dioxygenase that can be stabilized as described here. The present study provides a basis for further purification and structural characterization of the 8*R*-dioxygenase for comparison with lipoxygenases and other dioxygenases.

ACKNOWLEDGMENTS

Supported by MFR (03X-6523), a grant from the Swedish Institute to C. Su and by Uppsala University. We thank Dr. K.Wikvall for generous advice and discussion of the manuscript.

REFERENCES

1. Gardner, H.W. (1991) *Biochim. Biophys. Acta 1084*, 221–239.
2. Yamamoto, S. (1992) *Biochim. Biophys. Acta 1128*, 117–131.
3. Steczko, J., Donoho, G.P., Clemens, J.C., Dixon, J.E., and Axelrod, B. (1992) *Biochemistry 31*, 4053–4057.
4. Chen, X.-S., Kurre, U., Jenkins, N.A., Copeland, N.G., and Funk, C.D. (1994) *J. Biol. Chem. 269*, 13979–13987.
5. Boyington, J.C., Gaffney, B.J., and Amzel, L.M. (1993) *Science 260*, 1482–1486.
6. Minor, W., Steczko, J., Bolin, J.T., Otwinowski, Z., and Axelrod, B. (1993) *Biochemistry 32*, 6320–6323.
7. MacMillan, R.M., and Walker, E.H.R. (1992) *Trends Pharmacol. Sci. 13*, 323–330.
8. Brodowsky, I.D., and Oliw, E.H. (1992) *Biochim. Biophys. Acta 1124*, 59–65.
9. Brodowsky, I.D., Hamberg, M., and Oliw, E.H. (1992) *J. Biol. Chem. 267*, 14738–14745.
10. Brodowsky, I.D., and Oliw, E.H. (1993) *Biochim. Biophys. Acta 1168*, 68–72.
11. Hamberg, M., Zhang, L-Y., Brodowsky, I.D., and Oliw, E.H. (1994) *Arch. Biochem. Biophys. 309*, 77–80.
12. Brodowsky, I.D., Hamberg, M., and Oliw, E.H. (1994) *Eur. J. Pharmacol. 245*, 43–47.
13. Gerwick, W.H., Moghaddam, M., and Hamberg, M. (1991) *Arch. Biochem. Biophys. 290*, 436–444.
14. Hamberg, M., and Gerwick, W.H. (1993) *Arch. Biochem. Biophys. 305*, 115–122.
15. Song, W.-C., and Brash, A.R. (1991) *Science 253*, 781–784.
16. Song, W.-C., Funk, C.D., and Brash, A.R. (1991) *Proc. Natl. Acad. Sci. USA 90*, 8519–8523.
17. Oliw, E.H. (1989) *J. Biol. Chem. 264*, 17845–17853.
18. Matthew, J.A., Chan, H.W.-S., and Galliard, T. (1977) *Lipids 12*, 324–326.
19. Bradford, M.M. (1976) *Anal. Biochem. 72*, 248–254.
20. Hjertén, S., Pan, H., and Yao, K. (1982) in *Protides of the Biological Fluids*, 29th Colloquium 1981 (H. Peeters, ed.), pp. 15–25, Pergamon Press, Oxford.
21. Smith, W.L., and Lands, W.E.M. (1971) *J. Biol. Chem. 247*, 1038–1047.
22. Carroll, R.T., Muller, J., Grimm, J., Dunham, W.R., Sands, R.H., and Funk, M.O. (1993) *Lipids 28*, 241–244.
23. Percival, M.D. (1991) *J. Biol. Chem. 266*, 10058–10061.
24. Zhang, Y.-Y., Hamberg, M., Rådmark, O., and Samuelsson, B. (1994) *Anal. Biochem. 220*, 28–35.
25. Clissold, S.P. (1986) *Drugs 32 (suppl. 4)*, 46–59
26. Oliw, E.H. (1994) *Prog. Lipid Res. 33*, 329–354.
27. Guengerich, F.P. (1991) *J. Biol. Chem. 266*, 10019–10022.
28. Bowers, W.S., Hoch, H.C., Evans, P.H., and Katayama, M. (1986) *Science 232*, 105–106.
29. Mazur, P., Meyer, H.V., and Nakanishi, K. (1990) *Tetrahedron Lett. 31*, 3837–3840.
30. Mazur, P., Nakanishi, K., El-Zayat, A.A.E., and Champe, S.P. (1991) *J. Chem. Soc. Chem. Commun. 20*, 1486–1487.
31. Peng, Y.-L., Shirano, Y., Ohta, H., Hibino, T., Tanaka, K., and Shibata, D. (1994) *J. Biol. Chem. 269*, 3755–3761.
32. Funk, C.D. (1993) *Prog. Nucl. Acid Res. Mol. Biol. 45*, 67–98.

[Received July 18, 1994, and in revised form November 2, 1994; Revision accepted November 14, 1994]

Inhibition of Soybean Lipoxygenase-1 by Chain-Breaking Antioxidants

Mauro Maccarrone[a,c], Gerrit A. Veldink[c], Johannes F.G. Vliegenthart[c] and Alessandro Finazzi Agrò[b,*]

[a]IDI-IRCCS Unit of the [b]Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," I-00133 Rome, Italy and [c]Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht University, NL-3584 CH Utrecht, The Netherlands

ABSTRACT: The aim of this investigation was to determine whether chain-breaking antioxidants able to prevent lipid peroxidation can inhibit lipoxygenase-1 (EC 1.13.11.12). Therefore, the effects of ascorbic acid, 6-palmitoylascorbic acid and trolox on the enzyme activity were analyzed by means of Lineweaver–Burk double reciprocal plots and Yoshino's graphical method. The effect of these compounds on the formation of free radicals during lipoxygenase-1 reaction was investigated as well, by monitoring the enzymic formation of oxodienes. We present evidence that the chain-breaking antioxidants ascorbic acid, 6-palmitoylascorbic acid and trolox inhibit soybean lipoxygenase-1 in the micromolar concentration range (Ki 27, 3 and 18 μM, respectively). The inhibition is competitive, complete and reversible. All three compounds trap the free radicals formed during the lipoxygenase-catalyzed reaction, which might substantially contribute to their inhibitory ability. These findings can have physiological significance in the light of the lipoxygenase involvement in biomembrane remodelling.
Lipids 30, 51–54 (1995).

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are nonheme, nonsulfur–iron dioxygenases which act on fatty acids containing one or more 1,4-*Z,Z*-pentadiene moieties to form *Z,E*-conjugated hydroperoxides. Lipoxygenases from both animal and plant tissues have been purified and characterized (*cf.* Refs. 1–3 for reviews). These enzymes play several physiological roles (1–3), in particular the product hydroperoxides are precursors of specific regulatory molecules, such as leukotrienes and lipoxins in animals (3,4), and jasmonic acid and traumatin in plants (2,5). Since the discovery of the role of lipoxygenases in the biosynthesis of potent effectors critical in animal and plant physiology, extensive research was done to find efficient inhibitors of their activity. Most studies have used soybean lipoxygenase-1 (LOX), a homologue of mammalian lipoxygenases whose three-dimensional structure (6,7) and catalytic mechanism (8,9) have been characterized in detail. LOX activity is suppressed by several compounds, which act in different ways on the LOX-catalyzed reaction (10, 1). This paper describes the ability of the antioxidants ascorbic acid (12,13), 6-palmitoylascorbic acid (13) and trolox (a water-soluble analogue of α-tocopherol) (12) to competitively inhibit LOX activity. Keeping in mind the role of LOX in biomembrane alteration (14–16), it is suggested that chain-breaking antioxidants can protect cell membranes from lipid peroxidation (12,13,17) through the inhibition of the LOX-catalyzed oxygenation of polyenoic fatty acids.

*To whom correspondence should be addressed at Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," Via di Tor Vergata n. 135, I-00133 Rome, Italy.

Abbreviation: LOX, lipoxygenase-1.

MATERIALS AND METHODS

Chemicals were of the purest analytical grade. Linoleic acid, ascorbic acid and sodium dithionite were from Sigma (St. Louis, MO). 6-o-Palmitoyl-L-ascorbic acid was purchased from Fluka (Buchs, Switzerland) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Janssen (Geel, Belgium).

LOX was purified from soybean [*Glycine max* (L.) Merrill, var. Williams] seeds according to Finazzi Agrò *et al.* (18). The purified enzyme showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had a specific activity of 200 μmoles O_2.min^{-1}.mg $protein^{-1}$, assayed polarographically (19). In the inhibition experiments, LOX activity was determined spectrophotometrically by recording the formation of conjugated hydroperoxides at 234 nm (19). A stock solution of linoleic acid (30 μM), dissolved in 1% Tween-20 in water, was diluted to the appropriate final concentration in 0.1 M sodium phosphate buffer, pH 6.8, according to Lomnitski *et al.* (20). Dehydroascorbic acid was biosynthesized by incubating ascorbic acid with an excess of ascorbate oxidase, as reported Maccarrone *et al.* (21). The incubation mixture was filtered through centricon 30 microconcentrators (Amicon, Lexington, MA), in order to remove the enzyme molecules.

Copyright © 1995 by AOCS Press

The effect of inhibitors on linoleic acid oxygenation by LOX was analyzed by Lineweaver–Burk double reciprocal plots (20). Enzyme inhibition was also analyzed by Yoshino's graphical method (22) in order to distinguish partial from complete inhibitors. We observed that the residual activity, when related to the activity in the absence of the inhibitor, was independent of the sequence of reagents addition. Therefore, the reaction was started by addition of the substrate (in the range 15–120 μM) to the reaction mixture containing 15 nM LOX and different inhibitor concentrations, namely 0–350 μM ascorbic acid, 0–35 μM 6-palmitoylascorbic acid, 0–50 μM trolox or 0–100 μM sodium dithionite. Reversibility of inhibition (11) was assessed by assaying residual LOX activity after dialysis of the enzyme/inhibitor mixtures (1 mL) against 0.1 M sodium phosphate buffer, pH 6.8 (100 mL), at 4°C. The pH dependence of LOX inhibition was determined as reported (20,23), using 90 μM linoleic acid and 15 nM LOX. Progress curves of the oxygenation of 30 μM linoleic acid catalyzed by 15 nM LOX were recorded by following the formation of the hydroperoxide products at 234 nm (19). Polarographic measurements of LOX activity (19) always confirmed the spectrophotometric data.

The effect of inhibitors on the formation of side products of linoleic acid oxygenation was assessed by following the formation of total oxodienes (i.e., 13-oxo-octadeca-9,11-dienoic plus 13-oxo-trideca-9,11-dienoic acids) at 285 nm (10,24). The standard reaction mixture contained 90 μM linoleic acid, 15 nM LOX and different amounts of inhibitors, in 0.1 M sodium phosphate buffer, pH 6.8. The data reported in this paper are the mean of three independent determinations (coefficient of variation <7%).

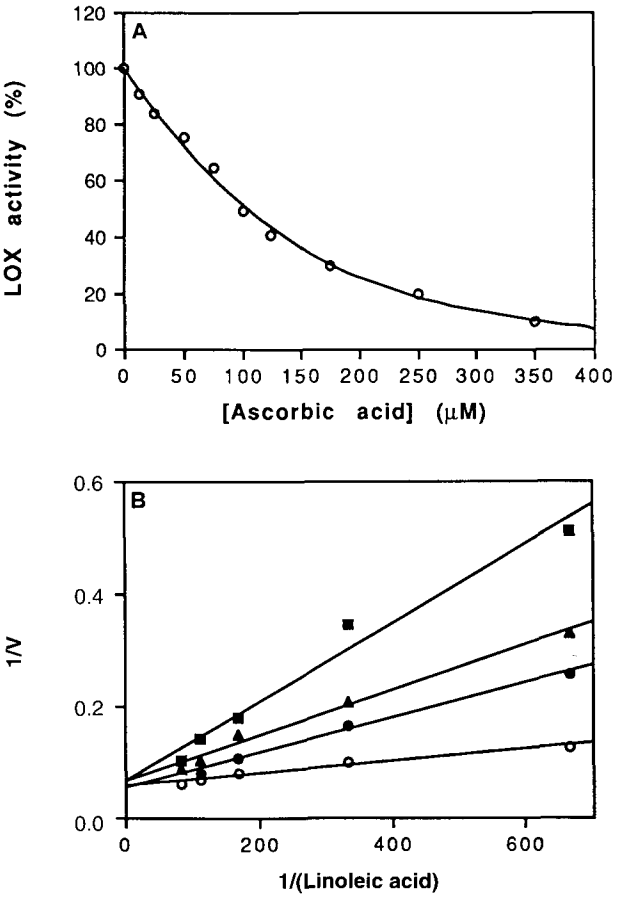


FIG. 1. A. Concentration-dependence of lipoxygenase-1 (LOX) inhibition by ascorbic acid. B. Lineweaver–Burk plots of linoleic acid oxygenation by LOX in the absence of inhibitors (open circles) or in the presence of 30 μM trolox (closed circles), 100 μM ascorbic acid (closed triangles) or 10 μM 6-palmitoylascorbic acid (closed squares). 1/V and 1/(Linoleic acid) values are expressed as $\mu M^{-1}.min$ and $\mu M^{-1} \times 10^4$, respectively.

RESULTS

Ascorbic acid suppressed LOX-catalyzed oxygenation of linoleic acid in a concentration-dependent manner (Fig. 1A). Double reciprocal plots for LOX in the presence and absence of ascorbic acid are shown in Figure 1B.

The Michaelis constant was found to be 19 μM for linoleate in the absence of inhibitor, a value which agrees well with previous determinations (8). Ascorbic acid proved to be a competitive inhibitor of LOX (Fig. 1B), its inhibition constant, Ki, being 27 μM. In order to ascertain whether ascorbic acid inhibition was complete or partial, Yoshino's fractional-velocity plot was drawn (22). This plot (not shown) was consistent with a complete competitive inhibition. Moreover, ascorbic acid acted as a reversible inhibitor, with LOX regaining full activity after four minutes of dialysis of enzyme/inhibitor mixtures (15 nM/100 μM) at 4°C. The ability of ascorbic acid to inhibit linoleic acid oxygenation by LOX was dependent on the pH of the reaction, increasing from pH 5.5 to pH 7.0, and then dropping down between pH 7.0 and 9.0 (Fig. 2A). Finally, ascorbic acid inhibited the formation of oxodienes during the LOX-catalyzed reaction in a concentration-dependent way (Fig. 2B).

The inhibition of oxodiene formation was greater than the inhibition of linoleic acid oxygenation (compare Fig. 1A with Fig. 2B). At variance with ascorbic acid, dehydroascorbic acid was unable to inhibit oxodiene formation and inhibited LOX activity to a lesser extent. In fact, 150 μM dehydroascorbic acid reduced linoleic acid oxygenation to 70% of the control value, compared to 38% obtained with 150 μM ascorbic acid. The same inhibition studies performed with ascorbic acid were done with the chain-breaking antioxidants 6-palmitoylascorbic acid and trolox. Both compounds were found to be complete, reversible and competitive inhibitors of LOX activity (Fig. 1B), Ki values being 3 and 18 μM, respectively (Table 1). Sodium dithionite, a nonspecific reductant, slowed down LOX-catalyzed reaction by acting as a noncompetitive inhibitor, with a Ki of 49 μM (Table 1).

Interestingly, sodium dithionite inhibition resulted in a lengthening of the lag phase (9,19) of linoleic acid oxygenation (Fig. 3A), at variance with ascorbic acid (Fig. 3B) and the other chain-breaking antioxidants (data not shown).

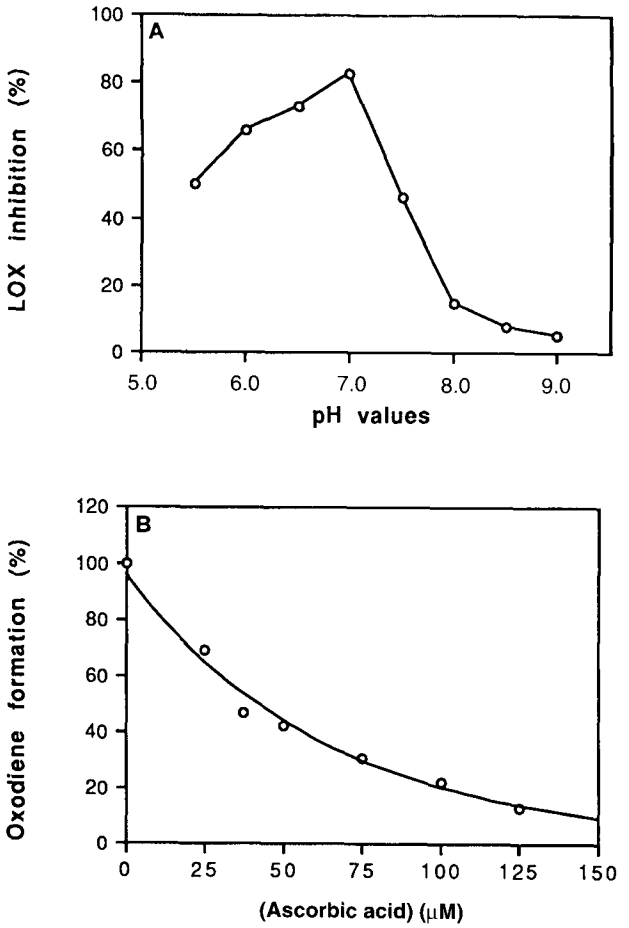


FIG. 2. A. Effect of pH on the inhibition of lipoxygenase-1 (LOX) activity by 250 μM ascorbic acid. B. Oxodiene formation in the presence of different amounts of ascorbic acid.

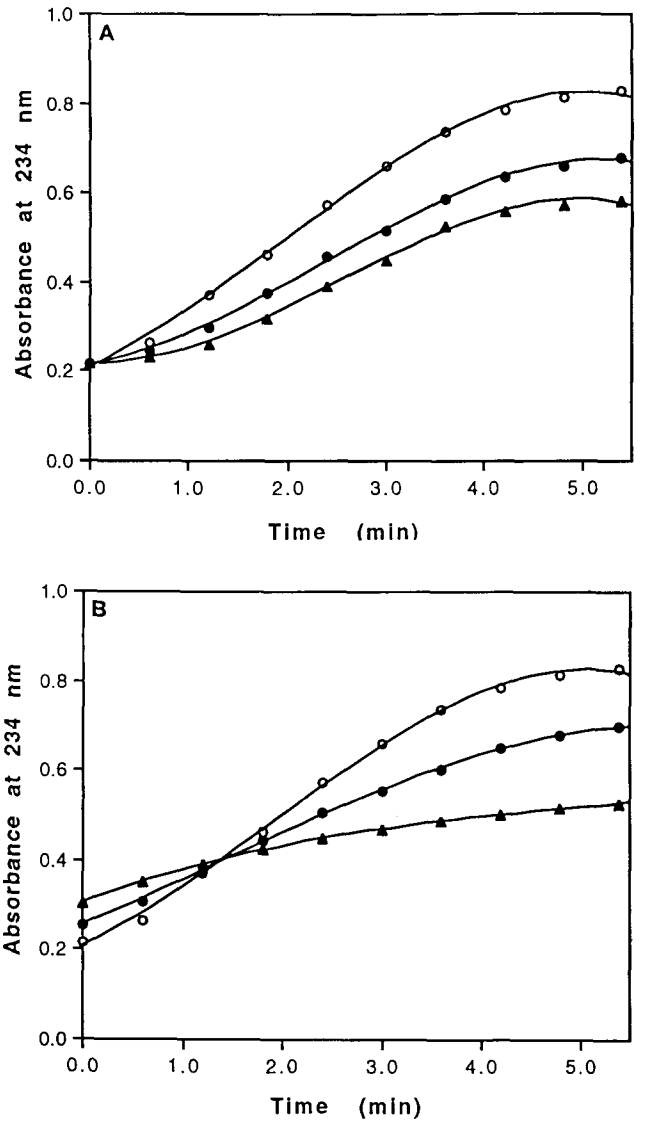


FIG. 3. A. Progress curves of LOX-catalyzed reaction, in the absence (open circles) or in the presence of 25 μM (closed circles) and 50 μM (closed triangles) sodium dithionite. B. Progress curves of LOX reaction in the absence (open circles) or in the presence of 50 μM (closed circles) and 100 μM (closed triangles) ascorbic acid. Abbreviation as in Figure 1.

TABLE 1
Inhibition of Lipoxygenase-1 by Chain-Breaking Antioxidants and Sodium Dithionite

Inhibitor	Inhibition type	Inhibition constant (Ki, μM)
Ascorbic acid	Competitive	27
6-Palmitoylascorbic acid	Competitive	3
Trolox	Competitive	18
Sodium dithionite	Noncompetitive	49

DISCUSSION

Oxygenation of linoleic acid catalyzed by soybean LOX is most effective at pH 9.0–10.0 (8,19). However, there was no inhibition of LOX under alkaline conditions (Fig. 2A). Conformational changes in the enzyme molecule might be responsible for the pH dependency of inhibition, a finding already reported for LOX (20,25). Therefore, experiments were performed at pH 6.8, where LOX retained high activity (26) and maximal inhibition was observed (Fig. 2A).

Several studies on soybean LOX showed that inhibitors can act through a number of mechanisms, e.g., by binding to sites other than the active site of the enzyme molecule (20), by preventing the formation of the activated Fe(III) form of LOX (27) or by reducing the catalytically active ferric enzyme to its inactive ferrous form through the formation of free radical metabolites (26). Here, ascorbic acid, 6-palmitoylascorbic acid and trolox are shown to act as competitive, complete and reversible inhibitors of LOX. Unlike other antioxidant inhibitors of LOX (26), the compounds under study did not reduce Fe(III) to inactive Fe(II), in fact the lag phase of the reaction (Fig. 3B) was not prolonged (9,10,19). Electron paramagnetic resonance spectroscopy (not shown) confirmed this finding, clearly indicating the lack of radical formation from the inhibitor molecules during LOX inhibition. At variance with the chain-breaking antioxidants, sodium dithionite acted as a noncompetitive inhibitor and caused a lengthening

of the lag phase of the reaction (Fig. 3A). This is consistent with sodium dithionite being a nonspecific reductant, which turns LOX into the inactive Fe(II) form (27). It can be suggested that the chain-breaking antioxidants might interact with the interior of the "funnel" leading to the active site (6), because enhancing the lipophilicity of the inhibitor made it more effective (Table 1). This hypothesis is in line with previous work on the competitive inhibition of LOX by *n*-alcohols, where longer alkyl chains were shown to increase the affinity of these compounds for the enzyme (28). Furthermore, ascorbic acid, 6-palmitoylascorbic acid and trolox are powerful radical-trapping antioxidants (12,13). Ascorbic acid strongly suppressed the formation of oxodienes (Fig. 2B), side products of linoleic acid oxidation which are indicative of the dissociation of free radicals from the active site (10,24). The same was found with 6-palmitoylascorbic acid and trolox (data not shown). These results suggest that the inhibitors can trap the radical intermediates escaping from LOX after hydrogen abstraction from linoleic acid, before enzymic oxygen insertion has occurred (8,10). Such radical-trapping ability might substantially contribute to the inhibition power of the compounds, as suggested also by the pH dependence (Fig. 2A). In fact, at neutral pH values, LOX produces more radicals than at pH 9.0, and trapping of these radicals, together with conformational changes of the enzyme (20), might improve LOX inhibition. The hypothesis that both hindrance of the active site and trapping of free radicals can contribute to LOX inhibition is also corroborated by the experiments with dehydroascorbic acid. The oxidized form of ascorbic acid was unable to reduce oxodiene formation (i.e., to trap radical intermediates), which resulted in a weaker inhibition of the LOX-catalyzed reaction. Finally, it seems noteworthy that trolox acted as competitive inhibitor of soybean LOX, at variance with its water-insoluble analogue α-tocopherol which inhibited the enzyme in a noncompetitive way (20). This difference resembles that of other compounds, e.g., retinol and β-carotene (20), and can be attributed to the different polarity of the two molecules.

In conclusion, evidence is presented that ascorbic acid, 6-palmitoylascorbic acid and trolox act as competitive inhibitors of soybean LOX. Thus, the effects of these chain-breaking antioxidants in protecting cell membranes by preventing lipid peroxidation can occur through the formation of an inactive complex with LOX, besides the trapping of free radicals. These results might have physiological significance in the light of lipoxygenase involvement in biomembrane alteration, both in animals (14) and in plants (15,16).

ACKNOWLEDGMENTS

The authors thank Dr. J. Pedersen (Department of Biology, University of Rome "Tor Vergata," Rome) for electron paramagnetic resonance spectroscopy measurements. This investigation was supported by E.U. grant SC1-0197.

REFERENCES

1. Vliegenthart, J.F.G., and Veldink, G.A. (1982) in *Free Radicals in Biology* (Pryor, W.A., ed.) pp. 29–64, Academic Press, New York.
2. Siedow, J.N. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42,* 145–188.
3. Yamamoto, S. (1992) *Biochim. Biophys. Acta 1128,* 117–131.
4. Samuelsson, B., Dahlem, S.E., Lindgren, S.E., Rouzer, C.A., and Serhan, C.N. (1987) *Science 237,* 171–176.
5. Hildebrand, D.F. (1989) *Physiol. Plant. 76,* 249–253.
6. Boyington, J.C., Gaffney, B.J., and Amzel, L.M. (1993) *Science 260,* 1482–1486.
7. Minor, W., Steczko, J., Bolin, J.T., Otwinowski, Z. and Axelrod, B. (1993) *Biochemistry 32,* 6320–6323.
8. Schilstra, M.J., Veldink, G.A., Verhagen, J., and Vliegenthart, J.F.G. (1992) *Biochemistry 31,* 7692–7699.
9. Schilstra, M.J., Veldink, G.A., and Vliegenthart, J.F.G. (1993) *Biochemistry 32,* 7686–7691.
10. Veldink, G.A., and Vliegenthart, J.F.G. (1991) in *Studies in Natural Products Chemistry* (Atta-ur-Rahman, ed.) Vol. 9, pp. 559–589, Elsevier, Amsterdam.
11. Cucurou, C., Battioni, J.P., Thang, D.C., Nam, N.H., and Mansuy, D. (1991) *Biochemistry 30,* 8964–8970.
12. Doba, T., Burton, G.W., and Ingold, K.U. (1985) *Biochim. Biophys. Acta 835,* 298–303.
13. Liu, Z.-L., Han, Z.-X., Chen, P., and Liu, Y.-C. (1990) *Chem. Phys. Lipids 56,* 73–80.
14. Kuhn, H., and Brash, A.R. (1990) *J. Biol. Chem. 265,* 1454–1458.
15. Kondo, Y., Kawai, Y., Hayashi, T., Ohnishi, M., Miyazawa, T., Itah, S., and Mizutani, J. (1993) *Biochim. Biophys. Acta 1170,* 301–306.
16. Maccarrone, M., Van Aarle, P.G.M., Veldink, G.A., and Vliegenthart, J.F.G. (1994) *Biochim. Biophys. Acta 1190,* 164–169.
17. Nagao, A., and Terao, J. (1990) *Biochem. Biophys. Res. Commun. 172,* 385–389.
18. Finazzi Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1973) *Biochim. Biophys. Acta 326,* 462–470.
19. Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1977) *Prog. Chem. Fats Other Lipids 15,* 131–166.
20. Lomnitski, L., Bar-Natan, R., Sklan, D., and Grossman, S. (1993) Biochim. *Biophys. Acta 1167,* 331–338.
21. Maccarrone, M., Rossi, A., D'Andrea, G., Amicosante, G., and Avigliano, L. (1990) *Anal. Biochem. 188,* 101–104.
22. Yoshino, M. (1987) *Biochem. J. 248,* 815–820.
23. Maccarrone, M., D'Andrea, G., Salucci, M.L., Avigliano, L., and Finazzi Agrò, A. (1993) *Phytochemistry 32,* 795–798.
24. Garssen, G.J., Vliegenthart, J.F.G., and Boldingh, J. (1971) *Biochem. J. 122,* 327–332.
25. Cucurou, C., Battioni, J.P., Daniel, R., and Mansuy, D. (1991) *Biochem. Biophys. Acta 1081,* 99–105.
26. Van der Zee, J., Eling, T.E., and Mason, R.P. (1989) *Biochemistry 28,* 8363–8367.
27. Sud'ina, G.F., Mirzoeva, O.K., Pushkareva, M.A., Korshunova, G.A., Sumbatyan, N.V., and Varfolomeev, S.D. (1993) *FEBS Lett. 329,* 21–24.
28. Kuninori, T., Nishiyama, J., Shirakawa, M., and Shimoyama, A. (1992) *Biochim. Biophys. Acta 1125,* 49–55.

[Received July 7, 1994, and in revised form October 26, 1994; Revision accepted November 21, 1994]

A Microsomal Membrane Component Associated with Iron Reduction in NADPH-Supported Lipid Peroxidation

Yoshiko Tampo and Masanori Yonaha*

Division of Environmental Hygiene, Hokkaido Institute of Pharmaceutical Sciences, Otaru, Hokkaido 047-02, Japan

ABSTRACT: This study was conducted to determine whether a factor responsible for reduced nicotinamide adenine dinucleotide phosphate (NADPH)-supported lipid peroxidation in rat liver microsomes is involved in iron reduction by cooperation with NADPH-cytochrome P450 reductase. Under anaerobic conditions, NADPH-dependent reduction of ferric pyrophosphate in microsomes was not dependent on cytochrome P450 levels and was not inhibited by carbon monoxide (CO). All of the iron complexes with chelators such as adenosine 5′-diphosphate, pyrophosphate, nitrilotriacetate, oxalate or citrate were reduced in microsomes, although in the reconstituted system containing purified NADPH-cytochrome P450 reductase little or no iron reduction was found. A cytochrome P450-free fraction from a cholate-solubilized preparation of microsomes after passage through a laurate sepharose column was required for reduction of iron pyrophosphate in the reconstituted system leading to lipid peroxidation. The iron reduction was not inhibited by CO and was destroyed by heat treatment or trypsin digestion of the fraction. All iron complexes were reduced in the presence of the fraction, using a reducing equivalent of NADPH *via* NADPH-cytochrome P450 reductase. The results indicate that a heat-labile component, which is probably a protein distinct from cytochrome P450, is associated with iron reduction responsible for lipid peroxidation in microsomes.
Lipids 30, 55–62 (1995).

Lipid peroxidation in biomembranes is mediated by free radical reactions leading to membranous degeneration. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-supported lipid peroxidation occurs in subcellular fractions of various tissues and has been extensively studied in rat hepatic microsomes (1–7). The peroxidation system requires iron chelates with a chelator such as adenosine 5′-diphosphate (ADP) or pyrophosphate (PPi) (1,2). The involvement of NADPH-cytochrome P450 reductase in the NADPH-supported lipid peroxidation was shown with the use of antibodies (3,8). The reduction of the iron complex is believed to be associated with initiation of lipid peroxidation. In the microsomes, the Fe^{3+}-ADP reduction occurs on the addition of NADPH alone (9). However, Fe^{3+}-ADP is not capable of interaction with isolated NADPH-cytochrome P450 reductase, and no iron reduction and no NADPH oxidation occur in the absence of exogenous Fe^{3+}-ethylenediaminetetraacetate (EDTA) (10).

In microsomes, ferric cytochrome P450 has been proposed to function as a propagator of lipid peroxidation much as is Fe^{3+}-EDTA in the reconstituted system consisting of Fe^{3+}-EDTA, Fe^{3+}-ADP, purified NADPH-cytochrome P450 reductase, microsomal lipids and NADPH (4). Recently, some evidence has also been provided showing that cytochrome P450 functions as a O_2^- generator resulting in reduction of the iron complex in the reconstituted peroxidation system (11), or is directly involved in reduction of the iron complex mediated *via* NADPH-cytochrome P450 reductase (12,13). NADPH oxidation that was accompanied by reduction of ferric iron, such as Fe^{3+} without a chelator, Fe^{3+}-ADP, and Fe^{3+}-citrate, was inhibited by carbon monoxide (CO), indicating involvement of cytochrome P450 (14). In contrast, some reports indicated noninvolvement of cytochrome P450 in NADPH-supported lipid peroxidation in microsomes, not inhibited by CO (7,15,16). Moreover, a different enzyme other than cytochrome P450 for the peroxidation has been suggested (8,16). Although traces of the hemoprotein were contained in the fraction used in one study (17), the involvement of another factor instead of cytochrome P450 in lipid peroxidation has also been suggested.

In this study, the cholate-solubilized fraction of microsomes was subjected to a laurate sepharose column to remove cytochrome P450. We report that the partially purified fractions, distinct from cytochrome P450, caused lipid peroxidation by reducing iron complexes through NADPH-cytochrome P450 reductase.

MATERIALS AND METHODS

Materials. NADPH was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), and 2-thiobarbituric acid and sodium cholate were obtained from E. Merck (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, MO), respectively. Sodium cholate was washed with ethanol on a Buchner funnel to remove a yellow pigment prior to use. Bathophenanthroline disulfonic acid 2Na salt (BPT) was obtained from

*To whom correspondence should be addressed.

Abbreviations: ADP, adenosine 5′-diphosphate; BPT, bathophenanthroline disulfonic acid; CO, carbon monoxide; EDTA, ethylenediaminetetraacetate; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NTA, nitrilotriacetate; PPi, pyrophosphate; TBARS, thiobarbituric acid-reactive substances.

Copyright © 1995 by AOCS Press

Wako Pure Chemicals Ind. Ltd (Osaka, Japan). The L-α-phosphatidylcholine (β-arachidonoyl-γ-stearoyl), trypsin (bovine pancreas, Type I), trypsin inhibitor (soybean, Type II-s) and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Company. The levels of peroxides in phosphatidylcholine, estimated by iodometry (18), were less than 0.5 mol%. All other chemicals employed were of commercial reagent-grade quality. Fe^{3+} was used as $FeCl_3$. Fe^{3+}-nitrilotriacetate (NTA) was prepared by dissolving NTA in dilute NaOH and adjusting to pH 7 followed by dissolution of $FeCl_3$. The other ferric chelates were prepared by dissolving $FeCl_3$ and chelator in H_2O. Concentration of iron complexes was expressed as iron in all experiments.

NADPH-cytochrome P450-reductase and microsomal lipids. NADPH-cytochrome P450 reductase was purified from rat liver microsomes according to the method of Yasukochi and Masters (19). Microsomal lipids were extracted from liver microsomes as described previously (17).

Cholate-solubilized microsomal fraction. Male Sprague-Dawley rats weighing about 250 to 300 g were killed by decapitation; the livers were removed, perfused with ice-cold saline, and homogenized in 10 vol of 0.25 M sucrose containing 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor. Microsomes were prepared by the calcium chloride aggregation method (20). The microsomal pellets from 4.5 g of livers were suspended by homogenizing in 45 mL of 0.15 M KCl containing 5 mM EDTA, and the suspension was centrifuged at 25,000 × *g* for 15 min. To remove EDTA and contaminated cytosol, the pellets were washed twice with 45 mL of 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M KCl and finally suspended in 50 mM Tris-HCl buffer (pH 7.5). Microsomal protein was estimated by the Biuret method (21). Differential solubilization of microsomes between 0.4 and 1.2% of sodium cholate was carried out as described previously (17), and the fraction was referred to as the cholate-cut fraction. Eight mL of the fraction was applied on a laurate AH-sepharose 4B column (1.5 × 21 cm) prepared by the method of Gibson and Schenkman (22), equilibrated with 5 mM sodium phosphate buffer (pH 7.5) containing 1.4% sodium cholate, 0.05 mM dithiothreitol and 20% (vol/vol) glycerol, and eluted with the equilibrating buffer. Protein in the eluates was measured by the method of Lowry *et al.* (23).

Preparation of lipid liposomes. All operations were carried out after purging with Ar, and sonication was done in ice-cold water. A solution of 3.5 mg microsomal lipids or phosphatidylcholine in chloroform was added to a small bottle, the chloroform removed, and the bottle sealed after inclusion of 1 mL of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.25 M NaCl purged with Ar. The bottle was placed in a Bransonic bath, and the lipids were dispersed by sonication for 0.5 min. Then the suspension was transferred to a thin-walled polycarbonate tube (1.6 × 7.9 cm), sealed with parafilm, and sonicated for 3 min. Sonication was repeated once again to yield an opalescent liquid. The levels of peroxides in phosphatidylcholine liposomes after sonication were 1.5 ± 0.4 mol% (n = 6).

Lipid peroxidation. NADPH-supported phospholipid peroxidation in the reconstituted systems was conducted as follows: The incubation mixture consisted of an aliquot (80 μL/mL) of eluate, liposomes of microsomal lipids (0.5 mg/mL) or 50 μM phosphatidylcholine, NADPH-cytochrome P450 reductase (0.06 nmol/mL), 0.2 mM Fe^{3+}-PPi (1:0.9, mol/mol), 0.15 M KCl and 25 mM Tris-HCl buffer, pH 7.5. After a 2-min temperature equilibration at 37°C, the reaction was initiated by adding 0.1 mM NADPH and was run at 37°C. Oxygen consumption was measured in a 1.5 mL cell at 37°C with a Clark electrode in a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs, OH) and was calibrated by the method of Misra and Fridovich (24). Thiobarbituric acid-reactive substances (TBARS) were quantified by the method of Ottolenghi (25), using the extinction coefficient at 535 nm of 156 $mM^{-1}cm^{-1}$(26), and were expressed in terms of malondialdehyde (MDA) production.

Reduction of iron complex. The incubation mixture contained iron complex, NADPH-cytochrome P450 reductase (0.06 nmol/mL), 0.1 mM NADPH, 0.15 M KCl, and 25 mM Tris-HCl buffer, pH 7.5. Reduction of iron was monitored by measuring an increase in absorbance at 534 nm on adding BPT (0.6 μmol) 20 s after the reaction was stopped with 0.1 mM *p*-chloromercuribenzoate. Ferrous was estimated using the extinction coefficient of 22,140 $M^{-1}cm^{-1}$ for Fe^{2+}-BPT (27).

Other assay. NADPH oxidation was monitored by measuring a decrease in absorbance at 340 nm at 37 °C. NADPH-cytochrome P450 reductase activity was measured using the extinction coefficient of 19.6 $mM^{-1}cm^{-1}$ of Yonetani (28). Cytochrome P450 and cytochrome b_5 were estimated by the methods of Omura and Sato (29) and Raw and Mahler (30), respectively.

RESULTS

Reduction of iron complexes in microsomes and by purified NADPH-cytochrome P450 reductase is shown in Table 1. In microsomes, all of the iron complexes were reduced, except Fe^{3+} without a chelator, consistent with the previous observation of little or no lipid peroxidation without chelator (2,7). Additionally, different reducibilities of iron complexes were found. No iron reduction of Fe^{3+}-ADP and Fe^{3+}-PPi occurred in the reconstituted system, which included the same levels of NADPH-cytochrome P450 reductase activity as that in microsomes, but Fe^{3+} without a chelator was reduced to some extent. Under the reconstituted conditions, Fe^{3+}-NTA, Fe^{3+}-citrate and Fe^{3+}-oxalate were also reduced, but these activities were much lower than those in the microsomes. In the presence of Fe^{3+}-NTA, the rates of lipid peroxidation for phosphatidylcholine liposomes in the reconstituted system were 44 ± 4 nmol O_2 /min/mL (oxygen consumption, n = 7) and 2.2 ± 0.5 nmol MDA/min/mL (TBARS production, n = 4), but Fe^{3+}-oxalate and Fe^{3+}-citrate showed little peroxidation activity (data not shown). Cholate treatment of microsomes diminished the cytochrome P450 content to 27% of the control, and

TABLE 1
Reduction of Iron or Iron Complexes in Microsomes and in the Reconstituted System[a]

System	Iron complex	Iron reduction (nmol Fe^{2+}/min/mL incubation mixture)
Microsomes	Fe^{3+}	n.d.[b] (6)[c]
	Fe^{3+}-ADP (1:17)	5.1 ± 0.7 (5)
	Fe^{3+}-PPi (1:0.9)	3.6 ± 0.5 (5)
	Fe^{3+}-NTA (1:1.1)	12.4 ± 1.7 (6)
	Fe^{3+}-oxalate (1:5)	1.6 ± 0.3 (4)
	Fe^{3+}-citrate (1:5)	9.4 ± 1.3 (5)
Reconstituted	Fe^{3+}	0.5 ± 0.1 (4)
	Fe^{3+}-ADP (1:17)	n.d. (6)
	Fe^{3+}-PPi (1:0.9)	n.d. (6)
	Fe^{3+}-NTA (1:1.1)	1.1 ± 0.4 (3)
	Fe^{3+}-oxalate (1:5)	0.2 ± 0.0 (3)
	Fe^{3+}-citrate (1:5)	0.2 ± 0.0 (3)

[a]The incubation mixture contained microsomes (0.5 mg protein/mL) or reduced nicotinamide adenosine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (0.06 nmol/mL) in the reconstituted system. All incubations contained 0.2 mM NADPH, 0.1 mM iron complex, 0.15 M KCl and 25 mM Tris-HCl buffer, pH 7.5 in a final volume of 1 mL. Iron reduction was measured as decribed in the Materials and Methods section. Data represent the means ± S.D. PPi, pyrophosphate; NTA, nitrilotriacetate; ADP, adenosine 5'-diphosphate.
[b]n.d., Not detected (lower than 0.05).
[c]The values in parentheses represent the number of determinations.

TABLE 2
Reduction of Fe^{3+}-PPi in Cholate-Treated Microsomes[a]

Treatment	Cytochrome P450 (nmol/mL incubation mixture)	Iron reduction) (nmol Fe^{2+}/min/mL incubation mixture)	
		Ar	CO
None	0.45 ± 0.01 (100)[b]	3.9 ± 0.3	3.9 ± 0.3
Cholate	0.12 ± 0.02 (27)	4.0 ± 0.3	3.9 ± 0.1
Cholate + glycerol	0.37 ± 0.03 (82)	4.1 ± 0.4	3.9 ± 0.2

[a]Aliquots of 20% sodium cholate were added to microsomes (20 mg/mL) suspended in 50 mM Tris-HCl buffer, pH 7.5, to yield a final concentration of 1.4% sodium cholate and 10 mg protein per mL, and the suspension was homogenized. The concentration of glycerol added was 20%. The incubation mixture contained 0.5 mg protein/mL, 0.2 mM NADPH, 0.1 mM Fe^{3+}-PPi (1:0.9, mol/mol), 0.15 M KCl and 25 mM Tris-HCl buffer (pH 7.5). Iron reduction was measured under a stream of Ar or CO, as described in the Materials and Methods section. Data represent the means ± S.D. of three determinations. Abbreviations as in Table 1; CO, carbon monoxide.
[b]The figures in parentheses indicate percent relative to "None."

the addition of glycerol reversed the content to 82% of the control (Table 2). Regardless of differences in the cytochrome P450 content in these microsomes, the reducing rates of Fe^{3+}-PPi under Ar were not altered, and no inhibition of the iron reduction by CO was observed. These findings strongly suggest that a component responsible for iron reduction, which is distinct from cytochrome P450 and exhibits different susceptibilities to CO, may be present in the microsomes.

The cholate-cut fraction of microsomes between 0.4 and 1.2% of sodium cholate was chromatographed on a laurate AH-sepharose 4B column, and the elution profile is given in

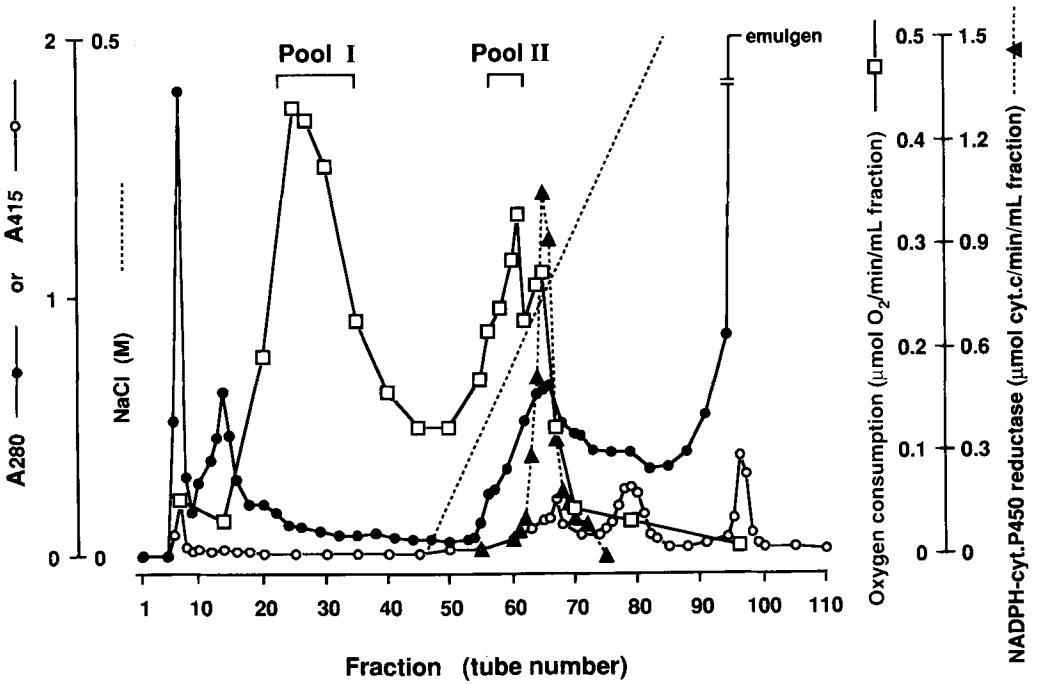


FIG. 1. Elution profile of the cholate-solubilized microsomes from a laurate AH-sepharose 4B column. Cholate solubilization of microsomes and laurate sepharose column chromatography were carried out as described in the Materials and Methods section. Volume of fractions was 3.4 mL. NADPH-supported lipid peroxidation was assayed in the reconstituted system containing 120 μL of eluate, 0.1 mM NADPH, 0.75 mg of microsomal lipid liposomes, 0.09 nmol NADPH-cytochrome P450 reductase, 0.2 mM Fe^{3+}-PPi (1:0.9), 0.15 M KCl and 25 mM Tris-HCl buffer, pH 7.5, in a final volume of 1.5 mL. Oxygen consumption was measured at 37°C. PPi, pyrophosphate; NADPH, reduced nicotinamide adenosine dinucleotide phosphate; Cyt.P450, cytochrome P450.

TABLE 3
Comparison of Various Indices in Cholate-Cut Fraction and Pooled Fractions

Assay[a]	Cholate-cut	Pool I	Pool II
Oxygen consumption[b]			
(nmol O_2/min/mL fraction)	1,800	260	210
(nmol O_2/min/mg protein)	210	4,300	680
MDA production[b]			
(nmol MDA/min/mL fraction)	120	20	15
(nmol MDA/min/mg protein)	14	330	48
NADPH-cytochrome P450 reductase			
(μmol cytochrome c/min/mL fraction)	1.7	<0.01	0.06
Cytochrome P450			
(nmol cytochrome P450/mL fraction)	5.1	<0.05	0.2
Cytochrome b_5			
(nmol cytochrome b_5/mL fraction)	2.6	<0.05	<0.05
Protein			
(mg protein/mL fraction)	8.5	0.06	0.31

[a]Each assay was conducted as described in the Materials and Methods section.
[b]The incubation mixture contained cholate-cut fraction (20 μL/mL) or pool (80 μL/mL), NADPH-cytochrome P450 reductase (0.06 nmol/mL) and microsomal lipid liposomes (0.5 mg/mL) in the reconstituted system. The incubation contained 0.1 mM NADPH, 0.2 mM Fe^{3+}-PPi (1:0.9, mol/mol), 0.15 M KCl, and 25 mM Tris-HCl buffer, pH 7.5, in a final volume of 1.5 mL. MDA, malondialdehyde; other abbreviations as in Table 1.

TABLE 4
Reduction of Iron or Iron Complexes in the Presence of Pool I in the Reconstituted System[a]

Iron complex	Iron reduction (nmol Fe^{2+}/min/mL incubation mixture)
Fe^{3+}	2.1 ± 0.1 (4)[b]
Fe^{3+}-ADP (1:17)	1.0 ± 0.1 (4)
Fe^{3+}-PPi (1:0.9)	1.3 ± 0.1 (12)
Fe^{3+}-NTA (1:1.1)	7.0 ± 0.4 (3)
Fe^{3+}-oxalate (1:5)	0.7 ± 0.2 (3)
Fe^{3+}-citrate (1:5)	1.2 ± 0.2 (3)

[a]The incubation mixture contained 80 μL of pool I, 0.06 nmol NADPH-cytochrome P450 reductase, 0.2 mM NADPH, 0.1 mM iron complex, 0.15 M KCl and 25 mM Tris-HCl buffer, pH 7.5 in a final volume of 1 mL. Iron reduction was measured as described in the Materials and Methods section.
[b]The values in parentheses represent the number of determinations. Data represent the means ± S.D. Abbreviations as in Table 1.

Figure 1. An aliquot of each fraction was added to the reconstituted system consisting of microsomal lipids, Fe^{3+}-PPi, NADPH-cytochrome P450 reductase, and NADPH, and lipid peroxidation was monitored. The first peak (pool I) exhibiting the principal activity was eluted with the equilibrating buffer, and subsequently, a lesser activity (pool II) was eluted with a linear 0–0.5 M NaCl gradient in the equilibrating buffer. The active peak eluting later than pool II appears to be produced by contaminating NADPH-cytochrome P450 reductase in the fraction. The same result of active peaks was seen when microsomes were not washed with EDTA, suggesting that a removable metal is not involved. The column was finally treated with 5 mM sodium phosphate (pH 7.5), containing 20% glycerol, 0.8% (wt/vol) Emulgen 911 and 1 M NaCl. About 70% of the protein loaded on the column was eluted prior to treatment with the last buffer. Four peaks of absorption at 415 nm, indicative of heme proteins, appeared over the total elution. The first peak contained neither cytochrome P450 nor cytochrome b_5, the second peak and the fourth peak contained both cytochrome P450 and a minor amount of cytochrome P420, and the third peak contained cytochrome b_5. NADPH-cytochrome P450 reductase, cytochrome P450, and cytochrome b_5 were not detected in pool I, and pool I revealed high specific activities of both oxygen consumption and TBARS production in the reconstituted system and low concentration of protein as compared with those in pool II or in the cholate-cut fraction (Table 3).

In the presence of Fe^{3+}-PPi and NADPH-cytochrome P450 reductase, NADPH oxidation was greatly stimulated by the addition of pool I to the reaction medium (Fig. 2A) and was accompanied by autoxidation of the Fe^{2+}-PPi formed (Fig. 2C). After further inclusion of phosphatidylcholine, lipid peroxidation monitored by O_2 consumption and TBARS production was highly promoted (Fig. 2D), but NADPH oxidation was not stimulated at all (Fig. 2B). After a 10-min incubation, the NADPH oxidation was somewhat reduced (Fig. 2B). This decrease may be due to partial inactivation of NADPH-cytochrome P450 reductase and/or a component in pool I by radicals generated during lipid peroxidation.

Phospholipid peroxidation activity of pool I completely disappeared on heat treatment at 90°C for 10 min and on trypsin digestion but not by treatment with heated trypsin (data not shown). Also, full phospholipid peroxidation occurred with trypsin in the presence of trypsin inhibitor. The result shows that a component exhibiting phospholipid peroxidation activity in pool I may be a heat-labile protein, in accord with the results of lipid peroxidation in microsomes and in the reconstituted system employing the fraction eluted from the Sephadex G-150 column (17).

Fe^{3+}-PPi was linearly reduced under a stream of Ar in the presence of NADPH-cytochrome P450 reductase, pool I, and NADPH, but the reduction was not caused without the addition of pool I (Fig. 3A). Reduced iron was not detected under air at all (data not shown). The iron reduction under Ar showed a dose-related response with an increase in added pool I, and the response was similarly observed under CO (Fig. 3B). Pool I was not replaced by the buffer employed in elution of pool I to reduce iron. Heat treatment and trypsin digestion of pool I caused no iron reduction. As compared with the reducing rates in the absence of pool I (Table 1), the addition of pool I enhanced Fe^{3+} reduction with or without chelators (Table 4).

DISCUSSION

Végh *et al.* (9) demonstrated iron reduction in microsomes in the reaction medium evacuated with Ar and suggested that the integrity of microsomal membranes was essential to NADPH-cytochrome P450 reductase activity to reduce ADP-Fe^{3+}, because the activity was greatly decreased by detergents. Cytochrome P450 has been proposed by some investi-

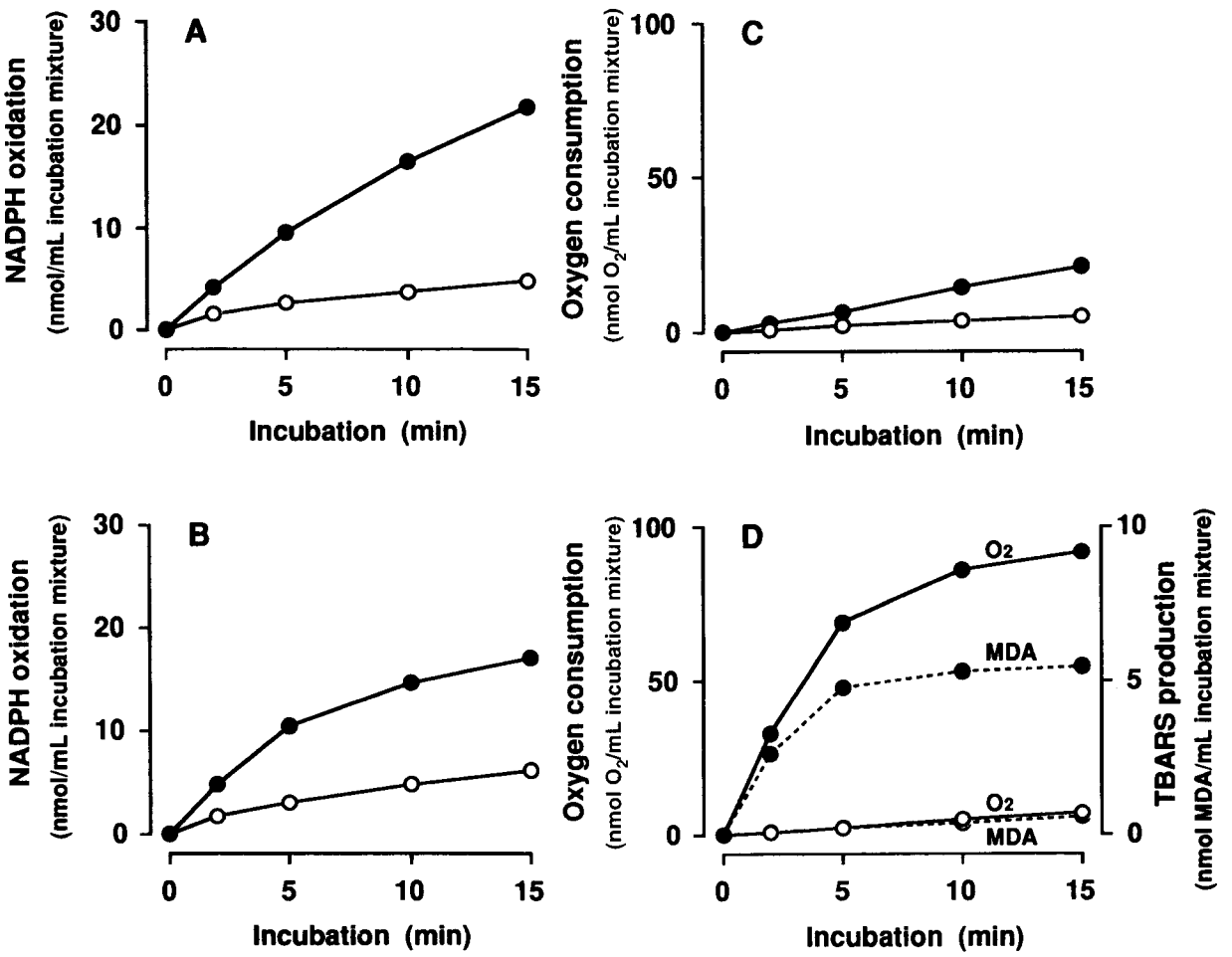


FIG. 2. NADPH oxidation and phospholipid peroxidation in the reconstituted systems. Complete incubation system contained 120 μL of pool I, 50-μM phosphatidylcholine liposomes, 0.1 mM NADPH, 0.09 nmol NADPH-cytochrome P450 reductase, 0.2 mM Fe^{3+}-PPi (1:0.9), 0.15 M KCl and 25 mM Tris-HCl buffer, p H 7.5, in a final volume of 1.5 mL. NADPH oxidation, oxygen consumption and malondialdehyde (MDA) production were assayed at 37°C as described in the Materials and Methods section. A and C, without phosphatidylcholine; B and D, with phosphatidylcholine. Data represent the means of four determinations for NADPH oxidation and the means of six determinations for oxygen consumption. ●, in the presence of pool I; ○, in the absence of pool I. TBARS, thiobarbituric acid-reactive substances; other abbreviations as in Figure 1.

gators (12,13) to be involved in iron reduction accompanied by lipid peroxidation in microsomes. Vile and Winterbourn (14) reported that NADPH oxidation in the presence of Fe^{3+} or Fe^{3+}-ADP at low levels in microsomes was inhibited by CO and the inhibition was reversed by an increase in added iron leading to competition with cytochrome P450 and suggesting involvement of the hemoprotein in the iron reduction. In the current study, iron reduction of Fe^{3+}-PPi in microsomes was not inhibited by CO and not influenced by alteration of cytochrome P450 content modulated by cholate and glycerol in microsomes. Thus, the direct assay of reduced iron appears to indicate no involvement of cytochrome P450.

Based on a study using the active fraction of lipid peroxidation in the reconstituted system, it has been demonstrated that NADPH-dependent iron reduction requires a component in pool I in the presence of NADPH-cytochrome P450 reductase. Some investigators (11-13) have studied the involvement of cytochrome P450 in phospholipid peroxidation in the reconstituted system composed of the iron complex, phospholipid liposomes, NADPH-cytochrome P450 reductase and NADPH; they added approximately 0.3 nmol cytochrome P450 to 1 mL of the reaction mixtures. The current assay of phospholipid peroxidation in the reconstituted system was conducted by the addition of 80 μL of pool I per mL of the reaction mixture. Since the detection limit of cytochrome P450 was 0.05 nmol per mL of pool I, the hemoprotein content corresponded to 0.004 nmol even at the maximum level in 1 mL of incubation mixture. Accordingly, even if present, the cytochrome P450 in the incubation mixtures is too low to promote lipid peroxidation.

Under a stream of Ar, purified NADPH-cytochrome P450 reductase alone could not reduce Fe^{3+}-PPi, in agreement with the result for Fe^{3+}-ADP described by others (10). Further addition of pool I, which was separated by a laurate sepharose column from cytochrome P450 and NADPH-cytochrome P450 reductase, was capable of reducing Fe^{3+}-PPi. The re-

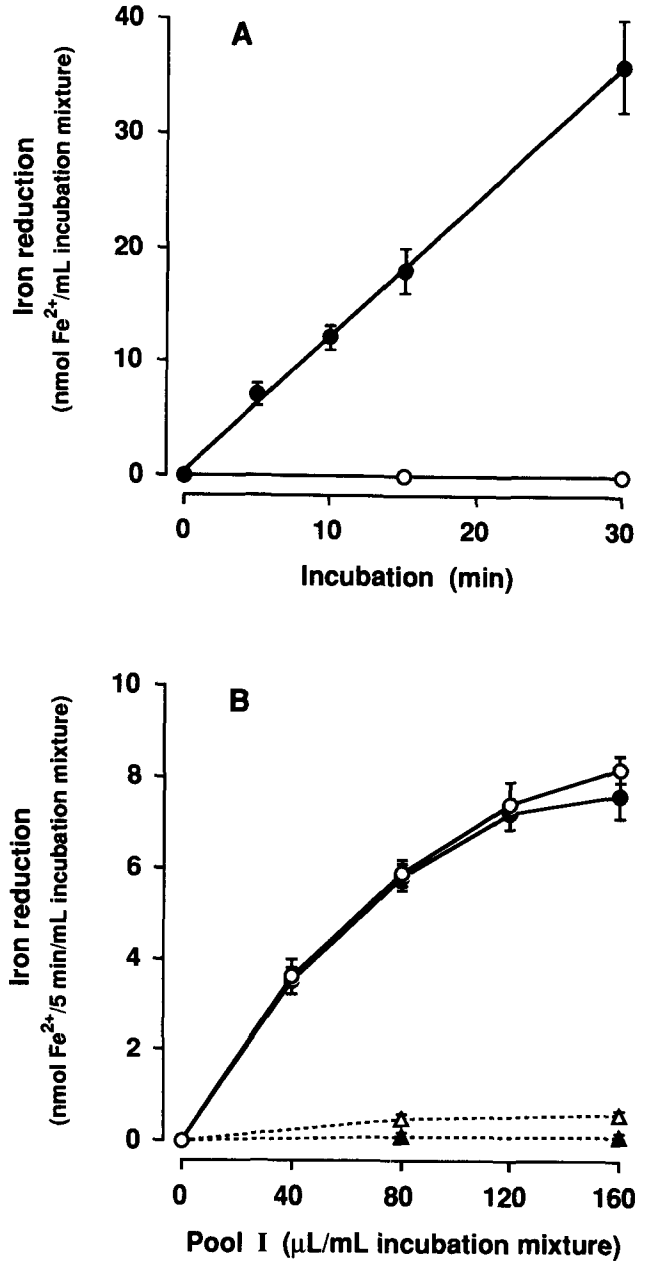


FIG. 3. Iron reduction under anaerobic conditions in the reconstituted systems. Complete system contained 80 μL of pool I, 0.1 mM NADPH, 0.06 nmol NADPH-cytochrome P450 reductase, 0.2 mM Fe^{3+}-pyrophosphate (1:0.9), 0.15 M KCl and 25 mM Tris-HCl buffer, pH 7.5, in a final volume of 1 mL. Heat treatment of pool I was carried out at 90°C for 10 min. Treatment of pool I (300 μL) with trypsin (5 μg) was conducted at 30°C for 25 min and stopped by adding trypsin inhibitor (10 μg). Iron reduction was measured as described in the Materials and Methods section. Data represent the means ± SD of four determinations. A, Time course under Ar. ●, complete system; ○, without Pool I. B, Dose response of pool I on iron reduction. ○, under Ar (pool I); ●, under carbon monoxide (CO) (pool I); △, (under Ar) trypsin digested pool I; ▲, (under Ar) heated pool I or the buffer employed in elution of pool I. Abbreviation as in Figure 1.

ducing activity of Fe^{3+}-PPi was destroyed by heat treatment or trypsin digestion of pool I and was not influenced by CO. The peroxidizing activity of pool I in the reconstituted system was also eliminated by heat treatment or trypsin digestion. These results indicate that a component in pool I is probably a protein, but clearly obviate the involvement of cytochrome P450 in iron reduction and phospholipid peroxidation in a NADPH-supported reconstituted system. The component in pool I was concentrated to about 8.5 times with cellulose acetate membrane (Ultracent-30; Tosoh Co. Ltd., Tokyo, Japan) (data not shown). The nature of the partially purified component has not been further determined. On the other hand, there are many enzymes in liver microsomes, other than cytochrome P450, which require NADPH-cytochrome P450 reductase and NADPH to function, e.g., cytochrome b_5 (31), fatty acyl coenzyme A desaturases (32), fatty acid elongase (33), homeoxygenase (34) or squalene epoxidase (35). However, no cytochrome b_5 was detected in pool I. Furthermore, since some of the above enzymes are solubilized with a detergent, such as deoxycholate (32) or Triton X-100 (35), without the loss of the enzyme activity, their possible involvement in iron reduction and phospholipid peroxidation may be excluded. For NADPH-dependent lipid peroxidation, the detergents are inhibitory in concentrations as low as 0.03% (36). Phospholipase A has been suggested to be involved at some stage in the alloxan- or copper-induced propagation of lipid peroxidation in rat liver microsomes (37). However, it has been shown that lipid peroxidation, catalyzed by a purified microsomal preparation in the presence of NADPH-cytochrome P450 reductase, NADPH and Fe^{3+}-PPi, was inhibited by *p*-bromophenacyl bromide, but inhibition of phospholipase A_2 required other reagents for inhibition (17). Thus, from the inhibitory effect of *p*-bromophenacyl bromide, it is conceivable that one or more nucleophilic functional group was involved.

The inclusion of *o*-phenanthroline or EDTA in the system containing NADPH-cytochrome P450 reductase and Fe^{3+}-ADP greatly stimulates NADPH oxidation which suggests iron reduction, although the absence of these chelators causes only a very slow rate of oxidation (38). The effect of these chelators is considered to be due to a newly formed iron chelate, which is an acceptable reducing equivalent *via* NADPH-cytochrome P450 reductase through NADPH. In the present experiments, free Fe^{3+} was poorly reduced in the system containing NADPH and NADPH-cytochrome P450 reductase, but under the conditions, Fe^{3+}-NTA was reduced highly to promote lipid peroxidation, whereas other chelators abolished or diminished the reducing rates of iron (Table 1). Thus, it appears that the effect of chelators on iron reduction causes production of the specific form of iron complex for interaction with the reductase. For instance, in the presence of NADPH-cytochrome P450 reductase and NADPH, Fe^{3+}-PPi or Fe^{3+}-ADP is markedly reduced in the presence of EDTA, to give rise to PPi (or ADP)-Fe^{3+}-EDTA complex, as compared with the reduction rate of Fe^{3+}-EDTA alone (39). In a manner similar to the effect of EDTA, it is conceivable that a component in the pooled fraction may cause a preferable configuration with the iron moiety to undergo interaction with reductase.

There was no evidence for reduction of free Fe^{3+} in microsomes, although free Fe^{3+} was reduced by NADPH-cy-

tochrome P450 reductase with or without pool I. This may reveal the occurrence of a component in microsomes which prevents interaction of free Fe^{3+} with NADPH-cytochrome P450 reductase and is not susceptible to chelated iron.

NADPH-supported lipid peroxidation in microsomes is promoted by the presence of iron chelates through an initiation reaction and a subsequent propagation reaction. The perferryl ion complex (1,4) has been proposed for the initiation of lipid peroxidation, but there is an argument, based on thermodynamic considerations, against its ability to abstract doubly allylic hydrogen (40). The ferrous dioxygen ferric complex (41) has also been proposed for the initiation. In this case, the 1:1 ratio of Fe^{2+} to Fe^{3+} is important (42). However, the mechanism may not always be supported by the fact that the effect of ferric ion is displaced by Al^{3+}, Pb^{2+} or Ca^{2+} (43,44). Recently, it has been suggested that an iron species appears to interact with anionic oxygen on phosphate moieties in phospholipids to promote lipid peroxidation which is competitively inhibited by Co(II) (45) or Mn(II) (46). In this case, a ferrous iron species would catalyze hydroperoxide breakdown by a site-specific mechanism on liposomal membranes producing alkoxyl radicals resulting in chain reactions (47). No stimulation of NADPH consumption was caused by the inclusion of phosphatidylcholine in the incubation system resulting in promotion of lipid peroxidation (Fig. 2), indicating that the Fe^{3+}-PPi reduction step is rate-limiting. Furthermore, phospholipid peroxidation in microsomes ceased rapidly when NADPH was depleted by addition of an α-ketoglutarate/glutamate dehydrogenase system (16). These findings provide evidence that the phospholipid peroxidation is greatly dependent on the iron reducing step.

Lipid peroxidation promoted in biomembranes may be not always associated with tissue degeneration. Vladimirov *et al.* (48) have proposed two effects of lipid peroxidation in normal tissues: membrane lipid exchange and cell division control. Some investigators reported that the peroxidized lipids formed may contribute to the turnover and remodeling of membranous phospholipids (49) and to the metabolizing of drugs (50). Thus, the occurrence of a component associated with iron reduction in microsomal membranes appears to suggest that phospholipid peroxidation *in vivo* may function in some physiological roles.

REFERENCES

1. Hochstein, P., and Ernster, L. (1963) *Biochem. Biophys. Res. Commun. 12,* 388–394.
2. Hochstein, P., Nordenbrand, K., and Ernster, L. (1964) *Biochem. Biophys. Res. Commun. 14,* 323–328.
3. Pederson, T.C., Buege, J.A., and Aust S.D. (1973) *J. Biol. Chem. 248,* 7134–7141.
4. Svingen, B.A., Buege, J.A., O'Neal, F.O., and Aust S.D. (1979) *J. Biol. Chem. 254,* 5892–5899.
5. May, H.E., and McCay, P.B. (1968) *J. Biol. Chem. 243,* 2288–2295.
6. Bell, J.G., Cowey, C.B., and Youngson, A. (1984) *Biochim. Biophys. Acta 795,* 91–99.
7. Ernster, L., Nordenbrand, K., and Orrenius, S. (1982) in *Lipid Peroxides in Biology and Medicine* (Yagi, K., ed.) pp. 55–79. Academic Press, New York.
8. Jansson, I., and Schenkman, J.B. (1977) *Arch. Biochem. Biophys. 178,* 89–107.
9. Végh, M., Marton, A., and Horváth, I. (1988) *Biochim. Biophys. Acta 964,* 146–150.
10. Tien, M., Morehouse, L.A., Bucher, J.R., and Aust, S.D. (1982) *Arch. Biochem. Biophys. 218,* 450–458.
11. Minakami, H., Arai, H., Nakano, M., Sugioka, K., Suzuki, S., and Sotomatsu, A. (1988) *Biochem. Biophys. Res. Commun. 153,* 973–978.
12. Morehouse, L.A., and Aust, S.D. (1988) *Free Radical Biol. Med. 4,* 269–277.
13. Sevanian, A., Nordenbrand, K., Kim, E., Ernster, L., and Aust, S.D. (1990) *Free Radical Biol. Med. 8,* 145–152.
14. Vile, G.F., and Winterbourn, C.C. (1988) *Arch. Biochem. Biophys. 267,* 606–613.
15. Baird, M.B. (1980) *Biochem. Biophys. Res. Commun. 95,* 1510–1516.
16. Davis, H.W., Suzuki, T., and Schenkman, J.B. (1987) *Arch. Biochem. Biophys. 252,* 218–228.
17. Yonaha, M., Tampo, Y., Clarke, W., Davis, H.W., and Schenkman, J.B. (1992) *Arch. Biochem. Biophys. 292,* 62–69.
18. Buege, J.A., and Aust, S.D. (1978) *Methods Enzymol. 52,* 302–310.
19. Yasukochi, Y., and Masters, B.S.S. (1976) *J. Biol. Chem. 251,* 5337–5344.
20. Schenkman, J.B., and Cinti, D.L. (1978) *Methods Enzymol. 52,* 83–89.
21. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) *J. Biol. Chem. 177,* 751–766.
22. Gibson, G.G., and Schenkman, J.B. (1978) *J. Biol. Chem. 253,* 5957–5963.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
24. Misra, H.P., and Fridovich, I. (1976) *Anal. Biochem. 70,* 632–634.
25. Ottolenghi, A. (1959) *Arch. Biochem. Biophys. 79,* 355–363.
26. Jordan, R.A., and Schenkman, J.B. (1982) *Biochem. Pharmacol. 31,* 1393–1400.
27. Carter, P. (1971) *Anal. Biochem. 40,* 450–458.
28. Yonetani, T. (1965) *J. Biol. Chem. 240,* 4509–4515.
29. Omura, T., and Sato, R. (1964) *J. Biol. Chem. 239,* 2370–2378.
30. Raw, I., and Mahler, H.R. (1959) *J. Biol. Chem. 234,* 1867–1873.
31. Rnoch, H.G., and Strittmatter, P. (1979) *J. Biol. Chem. 254,* 8976–8981.
32. Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M.J., Setlow, B., and Redline, R. (1974) *Proc. Nat. Acad. Sci. 71,* 4565–4569.
33. Ilan, Z., Ilan, R., and Cinti, D.L. (1981) *J. Biol. Chem. 256,* 10066–10072.
34. Schacter, B.A., Nelson, E.B., Marver, H.S., and Masters, B.S.S. (1972) *J. Biol. Chem. 247,* 3601–3607.
35. Ono, T., Ozasa, S., Hasegawa, F., and Imai, Y. (1977) *Biochim. Biophys. Acta 486,* 401–407.
36. Mishin, V.M., Pospelova, L.N., and Lyakhovich, V.V. (1976) *Arch. Biochem. Biophys. 174,* 630–636.
37. Beckman, J.K., Borowitz, S.M., Burr, I.M. (1987) *J. Biol. Chem. 262,* 1479–1484.
38. Noguchi, T., and Nakano, M. (1974) *Biochim. Biophys. Acta 368,* 446–455.
39. Tampo, Y., Onodera, S., and Yonaha, M. (1994) *Free Radical Biol. Med. 17,* 27–34.
40. Koppenol, W.H. (1994) in *Free Radical Damage and Its Control* (Rice-Evans, C.A. and Burdon, R.H. eds.) pp. 3–24, Elsevier, New York.

41. Bucher, J.R., Tien, M., and Aust, S.D. (1983) *Biochem. Biophys. Res. Commun. 111*, 777–784.
42. Samokyszyn, V.M., Miller, D.M., Reif, D.W., and Aust, S.D. (1990) in *Membrane Lipid Oxidation* (Vigo-Pelfrey, C., ed.) pp. 101–127, CRC Press, Boca Raton.
43. Aruoma, O.I., Halliwell, B., Laughton, M.J., Quinlan, G.J., and Gutteridge, J.M.C. (1989) *Biochem. J. 258*, 617–620.
44. Driomina, E.S., Sharov, V.S., and Vladimirov, Y.A. (1993) *Free Radical Biol. Med. 15*, 239–247.
45. Tampo, Y., and Yonaha, M. (1991) *Arch. Biochem. Biophys. 289*, 26–32.
46. Tampo, Y., and Yonaha, M. (1992) *Free Radical Biol. Med. 13*, 115–120.
47. Fujii, T., Hiramoto, Y., Terao, J., and Fukuzawa, K. (1991) *Arch. Biochem. Biophys. 284*, 120–126.
48. Vladimirov, Y.A., Olenev, V.I., Suslova, T.B., and Cheremisina, Z.P. (1980) *Adv. Lipid Res. 17*, 173–249.
49. Sevanian, A., Wratten, M.L., McLeod, L.L., and Kim, E. (1988) *Biochim. Biophys. Acta 961*, 316–327.
50. Capdevila, J., Estabrook, R.W., and Prough, R.A. (1980) *Arch. Biochem. Biophys. 200,* 186–195.

[Received August 4, 1994, and in revised form November 14, 1994; Revision accepted November 29, 1994]

Zinc Deficiency and Activities of Lipogenic and Glycolytic Enzymes in Liver of Rats Fed Coconut Oil or Linseed Oil

Klaus Eder and Manfred Kirchgessner*

Institut für Ernährungsphysiologie der Technischen Universität München Weihenstephan, 85350 Freising, Germany

ABSTRACT: In previous studies, zinc-deficient rats force-fed a diet with coconut oil as the major dietary fat developed a fatty liver, whereas zinc-deficient rats force-fed a diet with linseed oil did not. The present study was conducted to elucidate the reason for this phenomenon. In a bifactorial experiment, rats were fed zinc-adequate or zinc-deficient diets containing either a mixture of coconut oil (70 g/kg) and safflower oil (10 g/kg) ("coconut oil diet") or linseed oil (80 g/kg) ("linseed oil diet") as a source of dietary fat, and activities of lipogenic and glycolytic enzymes in liver were determined. In order to ensure adequate food intake, all the rats were force-fed. Zinc-deficient rats on the coconut oil diet developed a fatty liver, characterized by elevated levels of triglycerides with saturated and monounsaturated fatty acids. These rats also had markedly elevated activities of the lipogenic enzymes acetyl-CoA carboxylase, fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and citrate cleavage enzyme, whereas activities of malic enzyme and glycolytic enzymes were not different compared with zinc-adequate rats on the coconut oil diet. In contrast, rats receiving the linseed oil diet had similar triglyceride concentrations regardless of zinc status, and activities of lipogenic enzymes and glycolytic enzymes were not different between the two groups. Zinc-deficient rats fed either type of dietary fat exhibited statistically significant correlations between activities of FAS, G6PDH, 6PGDH and concentrations of saturated and monounsaturated fatty acids in liver. The concentrations of serum lipids were elevated in zinc-deficient rats fed either type of dietary fat. These results demonstrate that fatty liver in zinc-deficient rats on the coconut oil diet is caused by elevated activities of lipogenic enzymes, and not by disturbed lipid secretion from liver. Dietary linseed oil prevents both the elevation of lipogenic enzyme activity and fatty liver in zinc-deficient rats.
Lipids 30, 63–69 (1995).

A general problem in conventional zinc deficiency experiments is that only a few days after a zinc-deficient diet is administered rats largely reduce voluntary food intake (1–3). Therefore, rats fed a zinc-deficient diet suffer not only from zinc deficiency but generalized malnutrition. Thus, energy deficiency confounds the effects of zinc deficiency even in experiments including pair-fed control rats, particularly on parameters of lipid metabolism. In order to investigate the effects of zinc deficiency without distortion by the confounding effect of very low food intake, we fed rats a zinc-deficient diet by gastric tube (4–8). This force-feeding technique allows provision of sufficient nutrients and guarantees identical food intake and periodicity of feeding in control and zinc-deficient rats. In one of those studies, it was observed that rats force-fed a zinc-deficient diet containing predominately coconut oil as a source of dietary fat developed a fatty liver characterized by high concentrations of triglycerides with saturated and monounsaturated fatty acids (5), whereas rats force-fed a zinc-deficient diet containing oils rich in n-3 polyunsaturated fatty acids (PUFA) such as fish oil (5) or linseed oil (6) did not. The most likely reasons for the development of a fatty liver would be enhanced lipogenesis or disturbed secretion of lipids from liver into blood. Since concentrations of lipids in serum were elevated rather than reduced in rats force-fed zinc-deficient diets containing either type of fat (7,8), it is unlikely that disturbed secretion of lipids from liver into blood is the reason for fatty liver in the rats fed coconut oil. Therefore, we hypothesized that the rate of lipogenesis is enhanced in zinc-deficient rats fed a diet containing coconut oil but is not enhanced in rats fed a diet containing fish oil or linseed oil. To test this hypothesis, a bifactorial experiment was performed containing four groups of rats force-fed either zinc-deficient or zinc-adequate diets with a coconut oil/safflower oil mixture or linseed oil as source of dietary fat. To achieve information about the rate of lipogenesis, activities of hepatic enzymes involved in lipogenesis were determined: acetyl-CoA carboxylase (AcCx; E.C. 6.4.1.2) which provides malonyl-CoA for fatty acid synthesis, and fatty acid synthase (FAS) which is involved in chain elongation; glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGDH; E.C. 1.1.1.44) and malic enzyme (ME; E.C. 1.1.1.40) which provide NADPH; citrate cleavage enzyme (CCE; E.C. 4.1.3.8) which provides cytoplasmatic acetyl-CoA, and α-glycerophosphate dehydrogenase (αGPDH, E.C. 1.1.1.8) which provides α-glycerophosphate. Moreover, activities of two key

*To whom correspondence should be addressed.

Abbreviations: AcCx, acetyl-CoA carboxylase; ANOVA, analysis of variance; CCE, citrate cleavage enzyme; EFA, essential fatty acid; FAME, fatty acid methyl ester; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; αGPDH, α-glycerophosphate dehydrogenase; ME, malic enzyme; PFK, phosphofructokinase; PK, pyruvate kinase; 6PGDH, 6-phosphogluconate dehydrogenase; PUFA, polyunsaturated fatty acids.

Copyright © 1995 by AOCS Press

enzymes of glycolysis [phosphofructokinase (PFK), E.C. 2.7.1.11; pyruvate kinase (PK), E.C. 2.7.1.40] were determined to achieve information about glycolytic production of acetyl-CoA, which is the substrate for fatty acid synthesis.

MATERIALS AND METHODS

Animals and diets. Thirty-six male Sprague-Dawley rats weighing 121 ± 4 g were divided into four groups of nine rats each: zinc-adequate/coconut oil diet; zinc-deficient/coconut oil diet; zinc-adequate/linseed oil diet; zinc-deficient/linseed oil diet. The rats were housed individually in Macrolon cages. A daily 12-h light/dark cycle, a temperature of 23°C and 60% humidity were maintained. All the rats were fed four times per day (0800, 1300, 1800, 2300) by intragastric tube (5). The composition of the semisynthetic diets is given in Table 1. The basal diet contained 0.5 mg Zn/kg. The zinc-adequate diets were supplemented with 30 mg/kg Zn (as zinc sulfate). The coconut oil diets also contained safflower oil to provide essential fatty acids. The fatty acid composition of the dietary fats is given in Table 2. Diet slurries were freshly prepared before each feeding by mixing dry diet (92 g) with oil (8 g) and double distilled water (60 mL, with or without added zinc sulfate). Immediately before feeding, the slurry was warmed in a glass bottle at 50°C for a few minutes. The intragastric tube consisted of a 5-mL syringe connected with a slide catheter. During tube feeding, the conscious rat was handheld. The catheter was then inserted into the stomach of the rat, and the slurry was slowly injected. To avoid contamination, zinc-deficient rats were always fed before the control rats. Each rat received 4 mL of slurry per feeding (representing 2.9 g of food dry matter per feeding and 11.6 g of food dry matter per day). The rats had free access to drinking water [double-distilled water, supplemented with 0.14 g/L sodium chloride (suprapure) to adapt osmolarity to that of tap water].

TABLE 1
Composition of the Experimental Diets (g/kg diet)

Component	Coconut oil diet	Linseed oil diet
Casein (EDTA purified)	200	200
Corn starch	328	328
Sucrose	300	300
Coconut oil	70	—
Safflower oil	10	—
Linseed oil	—	80
Fiber (cellulose)	30	30
Mineral mixture[a]	40	40
Vitamin mixture[b]	20	20
DL-methionine	2	2

[a]Minerals per kg diet: Na_2HPO_4 • 2 H_2O 10.74 g; KH_2PO_4 8.20 g; KCl 6.00 g; $MgCl_2$ • 6 H_2O 3.40 g; $CaCO_3$ 13.6 g; $FeSO_4$ • 7 H_2O 248.8 mg; $CuSO_4$ • 7 H_2O 47.2 mg; $MnSO_4$ • 5 H_2O 46.1 mg; KI 9.0 mg; $NiSO_4$ • 6 H_2O 4.48 mg; Na_2MoO_4 • 2 H_2O 0.50 mg; $SnCl_2$ • 2H_2O 0.57 mg; Na_2SeO_3 • 5 H_2O 0.67 mg; $CrCl_3$ • 6 H_2O 0.51 mg; NH_4VO_3 0.23 mg; Na_2SiO_3 • 5 H_2O 1.51 mg; sugar to 40 g. EDTA, ethylenediaminetetraacetic acid.
[b]Vitamins per kg diet: 1.7 mg all-*trans* retinol; 7.5 µg cholecalciferol; 150 mg all-*rac*-α-tocopherol; 1 mg menadione sodium bisulfite; 5 mg thiamine • HCl; 10 mg riboflavin; 6 mg pyridoxine • HCl; 40 mg Ca pantothenate; 50 mg nicotinic acid; 1,000 mg choline chloride; 0.2 mg folic acid; 0.025 mg cyanocobalamin; sugar to 20 g.

TABLE 2
Fatty Acid Composition of the Dietary Fats (g/100 g fatty acids)

Fatty acid[a]	Coconut oil diet	Linseed oil diet
6:0	0.66	—[b]
8:0	10.10	—
10:0	6.44	—
12:0	40.61	—
14:0	14.45	—
16:0	7.94	5.74
18:0	3.26	3.43
18:1n-9	6.83	18.63
18:2n-6	9.94	15.29
18:3n-3	0.14	56.90
20:0	0.10	—
20:1n-9	0.10	—

[a]Each value represents the mean of five analyses.
[b]Indicates that fatty acid exists only in traces (< 0.1 g/100 g).

After 9 d, rats fed both types of zinc-deficient diet showed symptoms of zinc deficiency such as sparse and coarse hair, skin lesions around mouth, paws and eyes, ataxia and lethargy; they were obviously severely ill. Therefore, the experiment had to be terminated after 10 d of feeding. On the last day of the experiment, the rats were fed four times according to the regular schedule and were then additionally fed in the night (0500) to stimulate activity of lipogenic enzymes. Rats were then killed exactly 3 h after that feeding by decapitation under light anesthesia with diethyl ether.

Lipid analyses. Liver lipids were extracted with a hexane/isopropanol mixture (3:2, vol/vol, containing butylated hydroxytoluene as antioxidant) (9). For determination of total liver lipids, the solvent of a portion of the extract was evaporated, and the lipid mass was determined gravimetrically. For determination of liver total cholesterol and triglycerides, extract was dissolved in Triton X-100 as described (10). Total cholesterol and triglycerides were determined using enzymatic reagent kits obtained from Boehringer (Mannheim, Germany). Phospholipids of the extract were separated by high-performance liquid chromatography and quantified by their bound fatty acids (11).

Fatty acids of liver total lipids and dietary fats were converted into methyl esters by transesterification with boron fluoride/methanol reagent (12). Fatty acid methyl esters (FAMEs) were separated by gas chromatography using a Sichromat 2–8 gas chromatographic system (Siemens, Karlsruhe, Germany), fitted with a programmed temperature vaporizing injection system, a flame-ionization detector and a 50 m CP-Sil 88 capillary column (Chrompack, Middleburg, The Netherlands). FAMEs were identified by comparing retention times with those of individual purified standards, and quantified with heptadecanoic acid methyl ester as internal standard (11,13). Serum lipids (total cholesterol, triglycerides, phospholipids) were determined using an auto analyzer (Model 704; Hitachi, Tokyo, Japan) and commercial available enzymatic kit reagents (Boehringer).

Activities of liver enzymes. Approximately 4 g of liver was homogenized in 10 mL of 0.25 M Sucrose buffer (in 0.1 M phosphate buffer, pH 7.4) using a Potter-Elvehjem-homoge-

nizer. Homogenates were centrifuged (105,000 *g* for 1 h at 4°C), and the supernatants were used for enzyme assays. All the enzymes were assayed by spectrophotometric methods. G6PDH (14), 6PGDH (15) and ME (16) were determined by the rate of NADP reduction. In the assay of G6PDH, activity of 6PGDH was inhibited by maleimide (14). FAS was determined from the rate of malonyl-CoA-dependent NADPH oxidation (17), and AcCx was determined from NADH oxidation in an assay coupled with PK and lactate dehydrogenase (18). CCE was determined from NADH oxidation in an assay coupled with malate dehydrogenase (19). αGPDH was determined from α-glycerophosphate-dependent reduction of NAD (20). PFK determination was based upon the coupling of fructose-1,6-diphosphate formation to the oxidation of NADH with the use of aldolase, triosephosphate isomerase, and α-GPDH (21). PK was determined from NADH oxidation in an assay coupled with lactate dehydrogenase (22).

Zinc analysis and activity of alkaline phosphatase. Zinc concentration of serum was measured directly by aspirating a dilute solution (1:5) into the flame of an atomic absorption spectrophotometer (model 303; Perkin-Elmer, Überlingen, Germany). The activity of alkaline phosphatase in serum was determined using a commercial reagent kit (Boehringer) in an auto analyzer.

Statistical analysis. Treatment effects were analyzed by two-way analysis of variance (ANOVA) using the SPSS/PC + V3.0 software (SPSS, Chicago, IL). Classification factors were zinc supply and source of dietary fat, as well as interaction between those factors. Means of the four treatment groups were compared by the Student-Newman-Keuls multiple range test.

RESULTS

Weight gain and zinc status of the rats. Weight gain as well as activity of alkaline phosphatase and zinc concentration in serum are shown in Table 3. During the first 8 d of the experiment, the weight gain was nearly identical in the rats of all groups. Growth of the rats receiving the zinc-deficient diets then stopped, whereas weight gain of the rats receiving the zinc-adequate diets continued during the remainder of the experiment. The dietary fat did not influence mean daily growth rates which were higher for zinc-adequate rats than for zinc-deficient rats. Activity of alkaline phosphatase in serum was 65% lower in zinc-deficient rats fed the coconut oil diet than in zinc-adequate rats fed the coconut oil diet and 56% lower in zinc-deficient rats fed the linseed oil diet than in zinc-adequate rats fed the linseed oil diet. Serum zinc concentration was 78% lower in zinc-deficient rats fed the coconut oil diet than in equivalent control rats and 79% lower in zinc-deficient rats fed the linseed oil diet than in the equivalent control rats. Surprisingly, serum zinc concentration was significantly lower in zinc-adequate rats fed the linseed oil diet than in zinc-adequate rats fed the coconut oil diet, and there was a significant interaction between the factors zinc and fat on serum zinc concentration.

Liver and serum lipids. The effect of zinc deficiency on dry matter content and concentrations of total lipids and triglycerides was dependent on the type of dietary fat (Table 4). The zinc-deficient rats fed the coconut oil diet had a higher dry matter content and higher concentrations of total lipids and triglycerides in the liver than the control rats fed the coconut oil diet. In contrast, dry matter content and concentrations of total lipids and triglycerides in the zinc-deficient rats fed the linseed oil diet were not significantly different from those of the zinc-adequate rats fed the linseed oil diet. Concentration of total cholesterol in liver was not different between the four groups. Concentration of phosphatidylcholine was higher in zinc-deficient rats fed the coconut oil diet than in zinc-adequate rats fed the coconut oil diet, but was not significantly different between the two groups fed the linseed oil diet. Concentrations of phosphatidylethanolamine were not different between zinc-deficient and adequate rats fed both types of dietary fat.

TABLE 3
Weight Gain of the Rats During the Experiment, Activity of Alkaline Phosphatase and Zinc (Zn) Concentration in Serum at the End of the Experiment[a,b]

Parameter	Coconut oil diet		Linseed oil diet	
	Zn-adequate	Zn-deficient	Zn-adequate	Zn-deficient
Weight (g)				
Day 1	121 ± 4	121 ± 3	121 ± 4	121 ± 4
Day 6	146 ± 4	144 ± 4	147 ± 5	144 ± 3
Day 7	151 ± 4	147 ± 3	151 ± 4	147 ± 3
Day 8[f]	156 ± 3	151 ± 4	155 ± 4	150 ± 6
Day 9[f]	161 ± 3[c]	152 ± 6[d]	160 ± 5[c]	151 ± 6[d]
Day 10[f]	165 ± 4[c]	150 ± 7[d]	164 ± 6[c]	151 ± 6[d]
Day 11[f]	171 ± 3[c]	150 ± 5[d]	169 ± 5[c]	153 ± 7[d]
Mean daily weight gain (g/d)[f]	4.98 ± 0.34[c]	2.87 ± 0.51[d]	4.78 ± 0.38[c]	3.16 ± 0.59[d]
Alkaline phosphatase (U/L)[f]	706 ± 130[c]	251 ± 53[d]	763 ± 193[c]	339 ± 108[d]
Zinc concentration (μmol/L)[f,g,h]	15.3 ± 1.2[c]	3.36 ± 0.61[e]	13.5 ± 1.2[d]	2.91 ± 0.46[e]

[a]Values represent means ± SD with n = 9 for each group. Means with different superscript letters (c,d,e) within one row differ significantly by Student-Newman-Keuls test ($P < 0.05$).
[b]Significance of factors: [f]Zn: $P < 0.05$, [g]fat: $P < 0.05$, [h]Zn x fat: $P < 0.05$.

TABLE 4
Concentrations of Lipids in Liver and Serum of Zinc (Zn)-adequate and Zinc-deficient Rats[a,b]

Parameter	Coconut oil diet		Linseed oil diet	
	Zn-adequate	Zn-deficient	Zn-adequate	Zn-deficient
Liver (mg/g liver)				
Dry matter[f,g,h]	307 ± 4[c]	338 ± 28[d]	308 ± 8[c]	314 ± 10[c]
Total lipids[f,g,h]	69.0 ± 5.9[c]	113.2 ± 46.1[d]	63.5 ± 9.6[c]	70.8 ± 13.1[d]
Triglycerides[f,g,h]	34.2 ± 9.9[c]	86.1 ± 46.9[d]	25.0 ± 12.7[c]	36.6 ± 24.0[c]
Total cholesterol	2.10 ± 0.65	2.11 ± 0.55	2.04 ± 0.23	1.70 ± 0.23
Phosphatidylcholine[f]	11.88 ± 1.49[d]	14.82 ± 1.05[c]	13.40 ± 2.87[c,d]	15.70 ± 2.07[d]
Phosphatidylethanolamine	6.84 ± 0.68	7.29 ± 1.04	7.00 ± 0.80	7.69 ± 0.82
Serum (mg/dL)				
Triglycerides[f,g,h]	165.3 ± 57.8[c]	289.1 ± 125.2[d]	143.9 ± 65.0[c]	131.4 ± 38.6[c]
Total cholesterol[f]	55.0 ± 9.4[c]	77.2 ± 14.4[d]	49.1 ± 9.2[c]	68.1 ± 12.4[d]
Phospholipids[f,g]	122.6 ± 20.2[c,d]	196.2 ± 47.5[e]	103.4 ± 13.2[c]	146.2 ± 27.7[c]

[a]Values represent means ± SD with n = 9 for each group. Means with different superscript letters (c,d,e) within one row differ significantly by Student-Newman-Keuls test ($P < 0.05$).
[b]Significance of factors: [f]Zn: $P < 0.05$, [g]fat: $P < 0.05$, [h]Zn × fat: $P < 0.05$.

TABLE 5
Concentrations of Fatty Acids in Liver of Zinc (Zn)-adequate and Zinc-Deficient Rats[a,b]

Fatty acid	Coconut oil diet		Linseed oil diet	
	Zn-adequate	Zn-deficient	Zn-adequate	Zn-deficient
	(mg/g liver)			
Total fatty acids[f,g,h]	37.05 ± 5.12[c]	71.62 ± 35.71[d]	35.41 ± 7.48	40.27 ± 11.52
Total SFA[f,g,h]	15.54 ± 1.90[c]	36.20 ± 20.55[d]	12.54 ± 2.70	16.03 ± 4.89
10:0[f,g,h]	0.02 ± 0.01[c]	0.15 ± 0.14[d]	—[e]	—
12:0[f,g,h]	0.44 ± 0.19[c]	1.38 ± 1.24[d]	—	—
14:0[f,g,h]	1.14 ± 0.24[c]	3.15 ± 2.24[d]	0.23 ± 0.11	0.35 ± 0.16
16:0[f,g,h]	10.08 ± 1.41[c]	25.76 ± 15.74[d]	7.99 ± 2.17	10.63 ± 3.95
18:0[f,h]	3.52 ± 0.37[c]	5.41 ± 1.30[d]	4.05 ± 0.50[C]	4.80 ± 0.91[D]
20:0	0.01 ± 0.01[c]	0.04 ± 0.04[d]	0.02 ± 0.01	0.02 ± 0.01
22:0[g]	0.13 ± 0.01	0.14 ± 0.02	0.08 ± 0.01	0.08 ± 0.01
24:0[f]	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.02[C]	0.15 ± 0.01[D]
Total MUFA[f,g,h]	11.12 ± 2.72[c]	23.47 ± 14.49[d]	8.38 ± 3.50	9.49 ± 4.61
14:1[f,g,h]	0.08 ± 0.03[c]	0.33 ± 0.27[d]	0.06 ± 0.01	0.05 ± 0.01
16:1[f,g,h]	2.12 ± 0.74[c]	6.21 ± 4.21[d]	1.20 ± 0.68	1.72 ± 0.99
18:1[f,g,h]	8.77 ± 2.10[c]	16.77 ± 10.06[d]	6.95 ± 2.82	7.58 ± 3.60
20:1	0.06 ± 0.03	0.07 ± 0.04	0.06 ± 0.02	0.05 ± 0.02
24:1[g]	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.02	0.10 ± 0.01
Total PUFA[g]	10.40 ± 1.05[c]	11.95 ± 1.32[d]	14.58 ± 2.06	14.84 ± 2.53
Total n-6[g,h]	8.78 ± 0.89[c]	10.34 ± 1.26[d]	7.02 ± 0.70	6.46 ± 1.11
18:2[h]	3.88 ± 0.67[c]	4.98 ± 1.40[d]	4.07 ± 0.63	3.64 ± 0.68
18:3[f,g,h]	0.10 ± 0.02[c]	0.24 ± 0.12[d]	0.06 ± 0.02	0.07 ± 0.03
20:2[f]	0.16 ± 0.04[c]	0.24 ± 0.10[d]	0.18 ± 0.06	0.20 ± 0.09
20:3[g,h]	0.28 ± 0.04	0.30 ± 0.04	0.23 ± 0.03[C]	0.17 ± 0.04[D]
20:4[g]	3.94 ± 0.34	4.04 ± 0.58	2.33 ± 0.19	2.30 ± 0.40
22:4[g,h]	0.14 ± 0.03[c]	0.17 ± 0.03[d]	0.15 ± 0.04[C]	0.07 ± 0.05[D]
22:5[g]	0.28 ± 0.07	0.37 ± 0.12	—	—
Total n-3	1.62 ± 0.25	1.61 ± 0.13	7.46 ± 1.44	8.29 ± 1.66
18:3[g]	0.11 ± 0.04	0.11 ± 0.08	2.96 ± 1.13	3.40 ± 1.20
18:4[g,h]	—	—	0.10 ± 0.04	0.16 ± 0.10
20:3[g]	—	—	0.11 ± 0.02	0.10 ± 0.02
20:5[f,g,h]	0.07 ± 0.01	0.09 ± 0.03	1.98 ± 0.33[C]	2.41 ± 0.29[D]
22:5[g]	0.12 ± 0.03	0.11 ± 0.02	0.69 ± 0.17	0.71 ± 0.13
22:6[g]	1.31 ± 0.23	1.31 ± 0.13	1.62 ± 0.22	1.50 ± 0.32

[a]Values represent means ± SD with n = 9 for each group. Means were compared by pairs within one fat level. Means with different superscript letters (c,d; C,D) differ significantly ($P < 0.05$). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.
[b]Significance of factors: [f]Zn: $P < 0.05$, [g]fat: $P < 0.05$, [h]Zn × fat: $P < 0.05$.
[e]—: Concentration of fatty acid <0.01 mg/g.

TABLE 6
Activity[a] of Lipogenic and Glycolytic Enzymes in Liver[b,c]

Enzyme	Coconut oil diet		Linseed oil diet	
	Zn-adequate	Zn-deficient	Zn-adequate	Zn-deficient
Acetyl-CoA-carboxylase[f]	9.86 ± 5.28[d]	20.6 ± 11.5[e]	8.62 ± 3.23[d]	13.0 ± 2.6[d,e]
Fatty acid synthase[f]	2.30 ± 1.63[d]	5.59 ± 2.38[e]	3.22 ± 2.11[d]	4.02 ± 1.94[d,e]
Glucose-6-phosphate dehydrogenase[f]	14.2 ± 8.3[d]	29.1 ± 17.3[e]	11.2 ± 3.6[d]	20.3 ± 9.9[d,e]
6-Phosphogluconate dehydrogenase[f]	26.2 ± 6.3[d]	40.3 ± 11.6[e]	26.4 ± 6.9[d]	33.7 ± 7.2[d,e]
Malic enzyme	16.6 ± 2.5	14.5 ± 3.5	16.8 ± 4.0	15.1 ± 2.4
Citrate cleavage enzyme[f,g,h]	23.3 ± 4.3[d]	35.8 ± 9.6[e]	22.0 ± 4.9[d]	26.4 ± 3.2[d,e]
α-Glycerophosphate dehydrogenase[f]	184 ± 24[d]	150 ± 21[e]	185 ± 22[d]	169 ± 17[d,e]
Phosphofructokinase[f]	10.2 ± 1.45	11.3 ± 1.2	10.4 ± 1.0	11.3 ± 1.0
Pyruvate kinase[g]	1022 ± 193	971 ± 173	851 ± 170	847 ± 139

[a]Acetyl-CoA carboxylase and fatty acid synthase are expressed as nmoles NADPH oxidized/mg protein • min at 25°C; glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme are expressed as nmoles NADP reduced/mg protein • min at 25°C; citrate cleavage enzyme, phosphofructokinase and pyruvate kinase are expressed as nmoles NADH oxidized/mg protein • min at 25°C; α–glycerophosphate dehydrogenase is expressed as nmoles NAD reduced/mg protein • min at 25°C.
[b]Values represent means ± SD with n = 9 for each group. Means with different superscript letters (d,e) within one row differ significantly by Student-Newman-Keuls test ($P < 0.05$).
[c]Significance of factors: Zinc (Zn): $P < 0.05$, [g]fat: $P < 0.05$, [h]Zn x fat: $P < 0.05$.

All the rats had high concentrations of triglycerides in serum because they were fed 3 h before killing (Table 4). However, zinc-deficient rats fed the coconut oil diet had higher triglyceride concentrations than zinc-adequate rats fed the coconut oil diet whereas in the groups fed the linseed oil diet there was no difference between zinc-deficient and zinc-adequate rats. Concentration of total cholesterol was higher in both groups of zinc-deficient rats than in the equivalent control rats. Similarly, concentration of phospholipids in serum was higher in both groups of zinc-deficient rats than in the equivalent control rats. There was a significant effect of fat type and a significant interaction between the factors zinc and fat type on concentrations of triglycerides and phospholipids.

The effect of zinc deficiency on the concentrations of total fatty acids and most of the individual fatty acids in the liver was also dependent on the type of dietary fat (Table 5). The zinc-deficient rats fed the coconut oil diet had greatly elevated concentrations of saturated and monounsaturated fatty acids compared to the zinc-adequate rats fed the coconut oil diet, whereas concentrations of these fatty acids in the zinc-deficient rats fed the linseed oil diet were largely unchanged relative to the zinc-adequate rats fed the linseed oil diet. Zinc-deficient rats fed the coconut oil diet had also slightly elevated concentrations of n-6 PUFA relative to the zinc-adequate rats, while concentrations of n-3 PUFA were unchanged. In contrast, zinc-deficient rats fed the linseed oil diet had neither higher concentrations of total n-6 PUFA nor higher concentrations of total n-3 PUFA than zinc-adequate rats fed the linseed oil diet. However, concentration of eicosapentaenoic acid was higher in zinc-deficient rats than in zinc-adequate rats fed the linseed oil diet.

Activity of lipogenic and glycolytic enzymes. Zinc-deficient rats fed the coconut oil diet had significantly higher activities of AcCx, FAS, G6PDH, 6PGDH and CCE than zinc-adequate rats fed the coconut oil diet (Table 6). Activities of these enzymes were elevated in zinc-deficient rats fed the coconut oil diet in the range between 54 and 143%. In contrast, activities

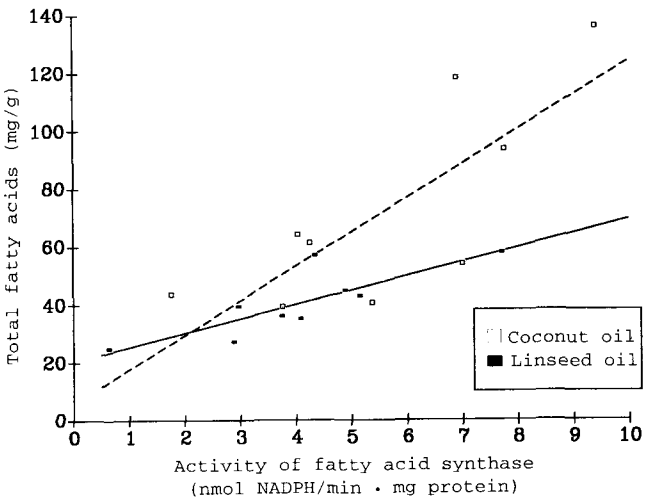


FIG. 1. Linear regression analysis between activity of fatty acid synthase and concentration of total fatty acids in liver of zinc-deficient rats fed coconut oil diet (....., Y = 6.0 + 11.80 • X, r = 0.61) and in zinc-deficient rats fed linseed oil diet (———, Y = 20.6 + 4.89 • X, r = 0.67).

of ME, PFK and PK were not different between these groups, and activity of αGPDH was lower in zinc-deficient rats fed the coconut oil diet than in the equivalent control rats.

In contrast, activity of none of the enzymes measured was significantly different between zinc-deficient rats fed the linseed oil diet and zinc-adequate rats fed the linseed oil diet. However, nominal values were higher in zinc-deficient rats than in zinc-adequate rats fed the linseed oil diet for AcCx (51%), FAS (25%), G6PDH (81%), 6PGDH (28%) and CCE (20%). ANOVA showed that the dietary fat influenced the activities of CCE and PK but not the activities of the other enzymes. There existed no statistical significant interaction between the factors zinc and fat on activity of any enzyme.

In the zinc-deficient rats fed both types of dietary fat, there existed significant ($P < 0.05$) linear correlations between activities of G6PDH, 6PGDH, FAS and concentrations of saturated and monounsaturated fatty acids in liver. In contrast, ac-

tivities of those enzymes did not correlate with concentrations of PUFA. There were no correlations between activities of ME, CCE and AcCx and concentrations of saturated, monounsaturated or PUFAs. Figure 1 shows the correlation between activity of FAS and concentration of liver total fatty acids in zinc-deficient rats fed both types of dietary fat.

DISCUSSION

The present study agrees with former studies (5,23) showing that rats force-fed a zinc-deficient diet with predominately saturated fatty acids accumulate triglycerides in liver, whereas rats fed a zinc-deficient diet with a highly polyunsaturated fat did not. Elevated activities of lipogenic enzymes in liver observed in the present study as well as elevated serum lipid concentrations in this and former studies (7,8) demonstrate that triglyceride accumulation is due to increased lipogenesis and not to disturbed secretion of lipids. Activity of AcCx, the key enzyme in lipogenesis (24), was twice as high in liver of zinc-deficient than in control rats fed the coconut oil diet. Unchanged activities of two key enzymes involved in glycolysis demonstrate that increased triglyceride concentration in liver is not due to enhanced production of acetyl-CoA by glycolysis.

There may be a connection between increased hepatic lipogenesis of zinc-deficient rats fed a highly saturated fat diet and disturbed metabolism of essential fatty acids. Zinc deficiency has been shown to produce similar clinical features in the rat as essential fatty acid (EFA) deficiency (25). Fatty liver that has been demonstrated in the present study in zinc-deficient rats fed a diet with a high saturated fat is also a symptom that occurs in EFA-deficient rats (26,27). Rats fed fat-free diets or diets containing hydrogenated fats also exhibit elevated activities of lipogenic enzymes in liver (28–30).

Oxygenation products derived from arachidonic acid were suggested to have a function in regulating activities of lipogenic enzymes (29,31). This was indicated by the observation that addition of safflower oil to a fat-free diet suppresses the activity of FAS and AcCx as well as G6PDH and 6PGDH in rat liver, whereas addition of safflower oil plus eicosa-5,8,11,14-tetraynoic acid (which inhibits many oxygenation reactions of arachidonic acid including cyclooxygenase, lipoxygenase and P-450 metabolism) did not (29,31). Fatty liver observed in zinc-deficient rats is not due to biochemical processes related to inhibited desaturation of linoleic acid because in the experiment presented here, zinc deficiency did not lower *in vitro* activity of Δ5- and Δ6-desaturases as well as levels of arachidonic acid in liver phospholipids and microsomes in the rats fed both types of dietary fat (Eder and Kirchgessner, unpublished data). On the other hand, the reason for the observation that zinc-deficient rats fed the linseed oil diet did not develop a fatty liver could be that feeding diets with a high level of α-linolenic acid causes synthesis of n-3 derived oxygenation products. The effects of those products are in several ways different from those derived from arachidonic acid (32). Because α-linolenic acid has been shown to be a more potent inhibitor of lipogenic enzymes (33) than linoleic acid, it is possible that 18:3 n-3 derivatives have a higher activity on inhibition of lipogenic enzymes than 18:2 n-6 derivatives.

One point that should be noted with respect to interpretation of fatty liver is that the ratio between feed intake and body weight at the end of the experiment was higher in zinc-deficient rats than in control rats. However, for two reasons it is likely that fatty liver is not the result of a higher relative feed intake. First, if higher relative feed intake would be the reason, fatty liver would be expected in zinc-deficient rats fed both dietary fats. Second, rats that voluntarily consume a higher quantity of diet than the rats of the present experiment do not gain a fatty liver (34).

In contrast with the present study, in zinc deficiency experiments in which rats voluntarily consumed the diet, with one exception (35) zinc deficiency did not elevate lipid concentrations in liver (36–39). This might be due to anorexia associated with zinc deficiency. Fasting or low food intake has been shown to markedly decrease activity of lipogenic enzymes (40). Moderately zinc-depleted rats that voluntarily consumed similar quantities of diet as the rats of the present study did not gain a fatty liver (39). This indicates that only severe zinc deficiency and high food intake at the same time increase lipogenesis in liver. Therefore, it can be concluded that fatty liver is not a physiologically-relevant effect because it is prevented by reduction of food intake in zinc deficiency.

Another point worthy of mention is that control rats fed the saturated fat diet and the highly unsaturated fat diet had similar activities of lipogenic enzymes in liver. This disagrees with studies indicating that PUFA are more potent in suppressing lipogenic enzyme activity than saturated fats (28,41). Possibly, the disparity in results is due to different feeding regimes because the eating period greatly influences lipogenesis in liver and adipose tissue. Meal-feeding, which is widely used in studies demonstrating the effect of fats on lipogenesis, has been shown to increase activity of lipogenic enzymes in comparison with *ad libitum* feeding (42). However, there have been no studies published on the effects of different dietary fats on lipogenic enzymes in rats force-fed the diet four times daily. As in the present study, rats fed diets high in either polyunsaturated or saturated fats were found to have equal degrees of inhibition of fatty acid synthesis (43).

REFERENCES

1. Todd, W.R., Elvehjem, C.A., and Hart, E.B. (1934) *Am. J. Physiol. 107*, 146–156.
2. Williams, R.B., and Mills, C.F. (1970) *Br. J. Nutr. 24*, 989–1003.
3. Chesters, J.K., and Quarterman, J. (1970) *Br. J. Nutr. 24*, 1061–1069.
4. Schülein, A., Kirchgessner, M., and Roth, H.P. (1992) *J. Anim. Physiol. a. Anim. Nutr. 67*, 157–169.
5. Eder, K., and Kirchgessner, M. (1994) *J. Nutr. 124*, 1619–1628.
6. Eder, K., and Kirchgessner, M. (1994) *Lipids 29*, 839 (1994).
7. Eder, K., and Kirchgessner, M. (1994) *Trace Elem. Electrolytes 11*, 55–60.
8. Eder, K., and Kirchgessner, M. (1994) *Trace Elem. Electrolytes 11*, 92–95.

9. Hara, A., and Radin, N.S. (1978) *Anal. Biochem. 90*, 420–426.
10. De Hoff, J.L., Davidson, L.H., and Kritchevsky, V. (1978) *Clin. Chem. 24*, 433–435.
11. Eder, K., Reichlmayr-Lais, A.M., and Kirchgessner, M. (1992) *J. Chromatogr. 598*, 33–42.
12. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5*, 600–608.
13. Eder, K., Reichlmayr-Lais, A.M., and Kirchgessner, M. (1991) *J. Chromatogr. 588*, 265–272.
14. Deutsch, J. (1983) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.) Vol. 3, pp. 190–197, Verlag Chemie, Weinheim.
15. Horecker, B.L., and Smyrniotis, P.Z. (1955) *Methods Enzymol. 1*, 323–327.
16. Hsu, R.Y., and Lardy, H.A. (1969) *Methods Enzymol. 8*, 230–235.
17. Roncari, D.A.K. (1981) *Methods Enzymol. 71*, 73–78.
18. Tanabe, T., Nakanishi, S., Hashimoto, T., Ogiwara, H., Nikawa, J-I., and Numa, S. (1981) *Methods Enzymol. 71*, 5–16.
19. Takeda, Y., Suzuki, F., and Inoue, H. (1969) *Methods Enzymol. 13*, 153–163.
20. Gonzalez-Cerezo, H., and Dalziel, K. (1975) *Methods Enzymol. 41*, 259–264.
21. Kemp, R.G. (1975) *Methods Enzymol. 42*, 71–77.
22. Staal, G.E.J., Koster, J.F., and Veeger, C. (1975) *Methods Enzymol. 42*, 182–186.
23. Eder, K., and Kirchgessner, M. (1993) *Z. Ernährungsw. 32*, 187–197.
24. Lane, M.D., Moss, J., and Polakis, S.E. (1974) *Curr. Top. Cell. Regulat. 8*, 139–195.
25. Bettger, W.J., Reeves, P.G., Moscatelli, E.A., Reynolds, G., and O'Dell, B.L. (1979) *J. Nutr. 109*, 480–488.
26. Sinclair, A.J., and Collins, F.D (1968) *Biochim. Biophys. Acta 152*, 498–510.
27. Bird, M.I., and Williams, M.A (1982) *J. Nutr. 112*, 2267–2278.
28. Musch, K., Ojakian, M.A., and Williams, M.A. (1974) *Biochim. Biophys. Acta 337*, 343–348.
29. Clarke, B.A., and Clarke, S.D (1982) *J. Nutr. 112*, 1212–1219.
30. Williams, M.A., Tamai, K.T., Hincenbergs, I., and McIntosh, D.J. (1972) *J. Nutr. 102*, 847–856.
31. Tomlinson, J.E., Nakayama, R., and Holten, D. (1988) *J. Nutr. 118*, 408–415.
32. Higgs, E.A., Moncada, S., and Vane, J.R. (1986) *Prog. Lipid Res. 25*, 5–11.
33. Odin, R.S., Adkins-Finke, B., Blake, W.L., Phinney, S.D., and Clarke, S.D. (1987) *Biochim. Biophys. Acta 921*, 379–391.
34. Stangl, G.I., Kirchgessner, M., Eder, K., and Reichlmayr-Lais, A.M. (1994) *Z. Ernährungswiss. 33*, 195–206.
35. Clejan, S., Maddaiah, V.T., Castro-Magana, M., and Collipp, P.J. (1981) *Lipids 16*, 454–460.
36. Fogerty, A.C., Ford, G.L., Dreosti, I.E., and Tinsley, I.J. (1985) *Nutr. Rep. Int. 32*, 1009–1019.
37. Huang, Y.-S., Cunnane, S.C., Horrobin, D.F., and Davignon, J. (1982) *Atherosclerosis 41*, 193–207.
38. Patel, P.B., Chung, R.A., and Lu, J.Y. (1975) *Nutr. Rep. Int. 12*, 205–210.
39. Cunnane, S.C. (1988) *Br. J. Nutr. 59*, 173–278.
40. Iritani, N., Nishimoto, N., Katsurada, A., and Fukuda, H. (1992) *J. Nutr. 122*, 28–36.
41. Toussant, M.J., Wilson, M.D., and Clarke, S.D. (1981) *J. Nutr. 111*, 146–153.
42. Leveille, G.A. (1970) *Fed. Proc. 29*, 1294–1301.
43. Wilson, M.D., Blake, W.L., Salati, M.L., and Clarke, S.D. (1990) *J. Nutr. 120*, 544–552.

[Received July 19, 1994, and in revised form October 27, 1994; Revision accepted November 11, 1994]

Synthesis and Metabolism of Sodium 3α,7α-Dihydroxy-25,26-Bishomo-5β-Cholane-26-Sulfonate in the Hamster

Takahiro Mikami[a,1], Erwin H. Mosbach[a,*], Bertram I. Cohen[a], Nariman Ayyad[a], Michiko Yoshii[b], Kenji Kihira[c] and Takahiko Hoshita[b]

[a]Department of Surgery, Beth Israel Medical Center, New York, New York, [b]Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine and [c]Department of Pharmaceutical Services, Hiroshima University Hospital, Hiroshima 734, Japan

ABSTRACT: This paper reports the chemical synthesis of a new bile acid analogue, namely sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate (bishomoCDC-sul) from chenodeoxycholic acid and describes its metabolism in the hamster. The structure of the new compound was confirmed by proton and carbon-13 nuclear magnetic resonance spectroscopy. After intravenous infusion of [^{3}H]-labeled sulfonate into bile fistula hamsters, it was extracted by the liver and secreted into the bile; more than 65% of the radioactivity was recovered in the bile within 1 h. Following intraduodenal administration of the [^{3}H]sulfonate and [^{14}C]chenodeoxycholyltaurine, both compounds were excreted into the bile more slowly; only 41 and 43% of the radioactivity, respectively, were recovered in the bile during the four-hour experimental period. In contrast, when the labeled compounds were injected into the terminal ileum, both the sulfonate and chenodeoxycholyltaurine were rapidly absorbed and secreted into the bile; 84 and 97%, respectively, of the radioactivity were recovered during a four-hour period. Chromatographic analysis demonstrated that in these short-term experiments most (>95%) of the sulfonate was secreted into the bile without biotransformation regardless of the route of administration. When infused intravenously at increasing doses, bishomoCDC-sul induced cholestasis at an infusion rate of 1 μmol/min/kg. These results suggest that sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate was absorbed from the terminal ileum by active transport, extracted by the liver, and secreted into the bile in a manner similar to that of the natural bile acids.
Lipids 30, 71–78 (1995).

We have synthesized and studied sulfonate analogues of the natural bile acids (1–6) in order to develop cholelitholytic agents that might be safe and have more persistent effects than chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA). These therapeutic agents are currently in use to dissolve cholesterol gallstones (7–9). In animal experiments, sulfonate analogues of CDCA (CDC-sul; sodium 3α,7α-dihydroxy-5β-cholane-24-sulfonate), UDCA (UDC-sul; sodium 3α,7β-dihydroxy-5β-cholane-24-sulfonate) and norursodeoxycholic acid (norUDC-sul) were efficiently absorbed from the terminal ileum by active transport and secreted into the bile in a manner similar to the natural bile acids (2,5). Orally-administered CDCA and UDCA are 7-dehydroxylated by intestinal microorganisms to form lithocholic acid (LCA) (10,11). This results in the loss of active drug from the enterohepatic circulation. It is also known that LCA is a hepatotoxic agent (12,13) and a promoter of colon cancer (14,15). In contrast, CDC-sul, UDC-sul and norUDC-sul were completely resistant to bacterial biotransformation (2,5).

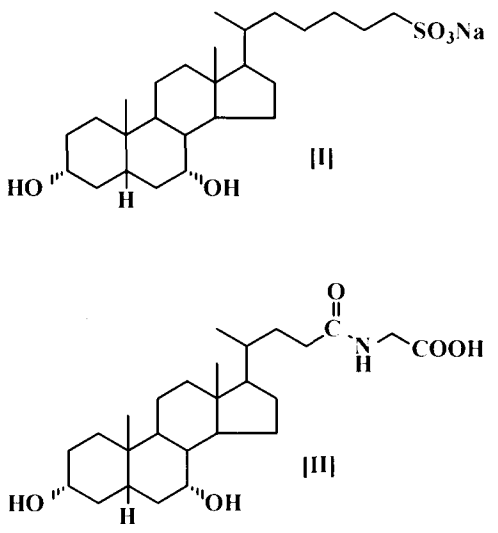
Recently, using sulfonate analogues of norchenodeoxycholic acid (norCDC-sul) and homochenodeoxycholic acid (homoCDC-sul), we demonstrated that side chain length affected the biological properties of the sulfonates. In dose response studies, intravenously infused homoCDC-sul produced cholestasis at a lower dose (0.75 μmol/min/kg) than norCDC-sul (2.0 μmol/min/kg) (4). It is known that bile acid hydrophobicity influences their cholestatic properties (16,17). By high-performance liquid chromatography (HPLC), homoCDC-sul is more hydrophobic than norCDC-sul. However, even though chenodeoxycholyltaurine (CDC-tau) is more hydrophobic than norCDC-sul, only the latter was cholestatic under the conditions employed (4). Sulfonate analogues do not possess a peptide bond in the side chain. In addition, the side chains of both sulfonates (norCDC-sul, homoCDC-sul) are shorter than that of CDC-tau. Therefore, we hypothesized that the cholestatic effect of sulfonate analogues will be reduced in compounds possessing the same side chain length as the natural conjugated bile acids. To test this hypothesis, we synthesized the sulfonate analogue of bishomochenodeoxycholic acid (bishomoCDCA), namely sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate (bishomoCDC-sul) (Scheme 1), in which the

[1]Permanent address: Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima, 734 Japan.

*To whom correspondence should be addressed at Department of Surgery, Beth Israel Medical Center, First Avenue at 16th St., New York, NY 10003.

Abbreviations: BishomoCDCA, bishomochenodeoxycholic acid; bishomoCDC-sul, sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate; CDCA, chenodeoxycholic acid; CDC-gly, chenodeoxycholylglycine; CDC-tau, chenodeoxycholyltaurine; CDC-sul, sodium 3α,7α-dihydroxy-5β-cholane-24-sulfonate; HPLC, high-performance liquid chromatography; ^{13}C NMR, carbon-13 nuclear magnetic resonance; homoCDC-sul, sodium 3α,7α-dihydroxy-25-homo-5β-cholane-25-sulfonate; ^{1}H NMR, proton nuclear magnetic resonance; LCA, lithocholic acid; norCDC-sul, sodium 3α,7α-dihydroxy-24-nor-5β-cholane-23-sulfonate; norUDC-sul, sodium 3α,7β-dihydroxy-24-nor-5β-cholane-23-sulfonate; TLC, thin-layer chromatography; UDCA, ursodeoxycholic acid; UDC-sul, sodium 3α,7β-dihydroxy-5β-cholane-24-sulfonate.

Copyright © 1995 by AOCS Press



SCHEME 1

length of the side chain is the same as that of chenodeoxycholylglycine (CDC-gly) (Scheme 1), and studied its metabolism and cholestatic properties in the hamster.

MATERIALS AND METHODS

General. Melting points were determined on a Thermolyne melting point apparatus (Dubuque, IA) and are not corrected. Proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were determined on a JEOL (Tokyo, Japan) JMN-FX-400 spectrometer (400 MHz) in CD_3OD solution using tetramethylsilane as internal standard. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} plates (0.2 mm thickness; EM Science, Darmstadt, Germany); a 10% ethanolic solution of phosphomolybdic acid was used to visualize the spots. HPLC was carried out on a Varian (Palo Alto, CA) model 5020 liquid chromatograph with a refractive index detector (HP1047A; Hewlett-Packard, Avondale, PA) and an ultraviolet detector (Waters 480 variable wavelength detector; Millipore, Milford, MA). A NOVA-C_{18} 4μ column (Waters Associates) was used in a Waters Z-module (solvent; methanol/water, 75:25, pH adjusted to 5.37 with phosphoric acid, flow rate 1.0 mL/min). Radioactivity was measured on a Beckman LS 3801 liquid scintillation system (Beckman Instruments, Fullerton, CA) with automatic quench correction, using Aquasol-2 (NEN Research Products, Boston, MA) as the scintillation fluid.

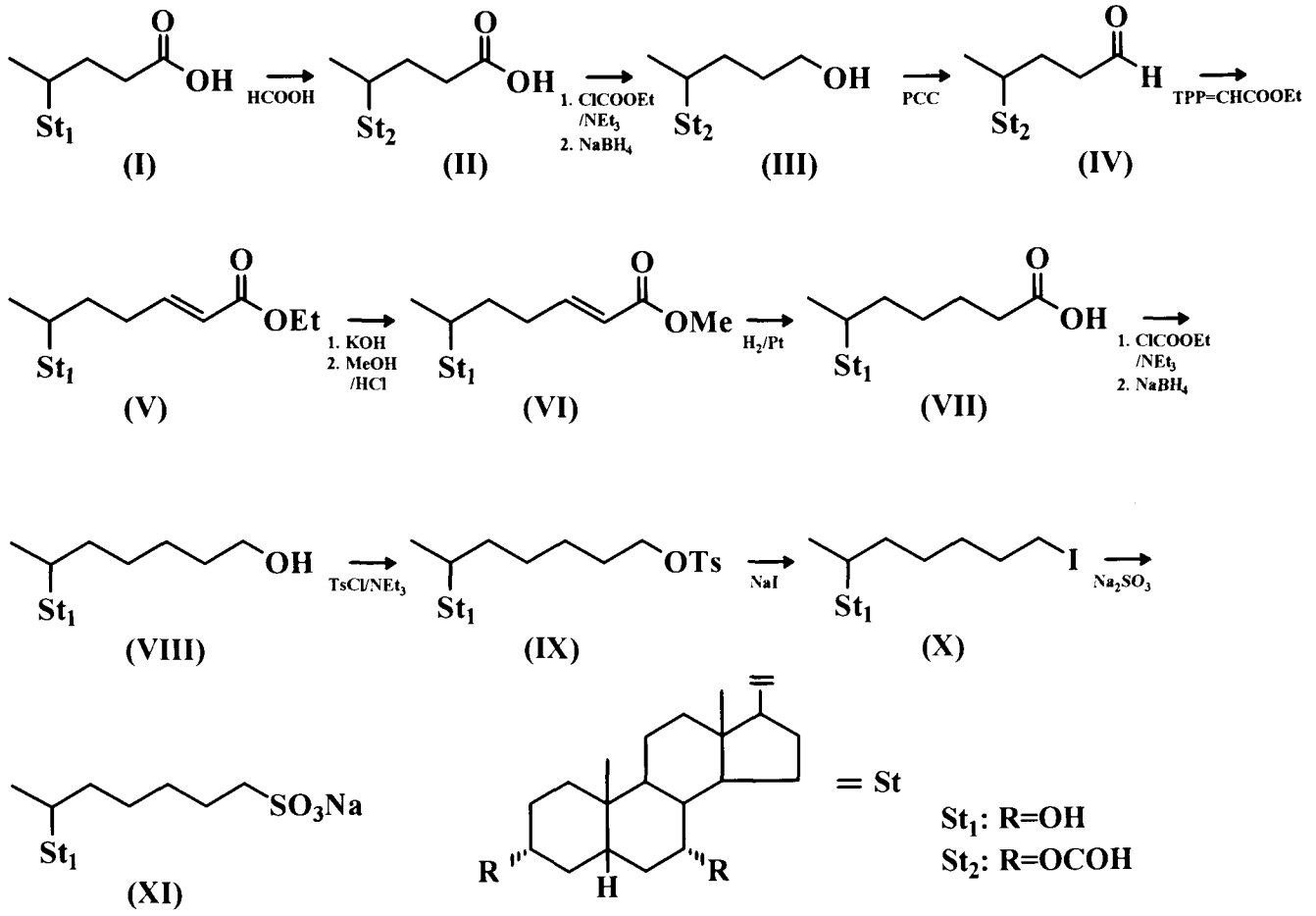
Synthesis. 3α,7α-Diformoxy-5β-cholan-24-ol (Scheme 2, **III***).* 3α,7α-Diformoxy-5β-cholan-24-oic acid (Scheme 2, **II**) was synthesized from CDCA (Scheme 2, **I**) as described previously (18). Triethylamine (15 mL) and 10 mL of ethyl chloroformate were added to a tetrahydrofuran (150 mL) solution of (II) (20.0 g). This solution was stirred at 0°C for 2 h. One-hundred and fifty mL of a 10% aqueous solution of sodium borohydride were added and stirred at room temperature for three hours. The solution was diluted with water, acidified with 1 N HCl and extracted with ethyl acetate. After evaporation, the residue was purified on a silica gel G 60 column (300 g, 35–70 mesh; ASTM; Merck, Darmstadt, Germany) using increasing proportions of ethyl acetate in benzene as eluent. The fractions containing 3α,7α-diformoxy-5β–cholan-24-ol (Scheme 2, **III**) were collected and evaporated; 12.7 g of an oily residue (**III**) was obtained.

3α,7α-Diformoxy-5β-cholan-24-al (Scheme 2, **IV***).* Sodium acetate (1.2 g) and 8.6 g of pyridinium chlorochromate were added to a solution of 3α,7α-diformoxy-5β-cholan-24-ol (Scheme 2, **III**) (12.7 g) in methylene chloride (150 mL). This solution was stirred at room temperature for two hours. The reaction mixture was diluted with 250 mL of ether and applied to a Florisil (150 g) column. After elution with ether, evaporation of the appropriate fractions gave 9.5 g of 3α,7α-diformoxy-5β-cholan-24-al (Scheme 2, **IV**). This residue was used for the next reaction without purification.

Methyl 3α,7α-dihydroxy-27-nor-5β-cholest-24-en-26-oate (Scheme 2, **VI***).* 3α,7α-Diformoxy-5β-cholan-24-al (Scheme 2, **IV**) (9.5 g) was dissolved in 50 mL of dry benzene and added to a solution of (carbethoxymethylene)triphenylphosphorane (11.5 g) in 25 mL of dry benzene. The reaction mixture was refluxed for 7 h. After dilution with water and acidification with 1 N HCl, the reaction mixture was extracted with ethyl acetate. The residue (Scheme 2, **V**) was dissolved in 100 mL of 5% methanolic KOH, and the solution was refluxed for 1.5 h. The hydrolyzate was extracted with ethyl acetate after dilution with water and acidification with 1 N HCl. The methyl ester was prepared by addition of 100 mL of methanol/HCl (100:3). The solution was diluted with water and extracted with ethyl acetate. The residue was purified on a silica gel G 60 column (300 g) using increasing proportions of ethyl acetate in benzene. The fractions containing methyl 3α,7α-dihydroxy-27-nor-5β-cholest-24-en-26-oate (Scheme 2, **VI**) were collected and evaporated; 2.0 g of (**VI**) were obtained. ^{1}H NMR 0.69(3H, *s*, 18–CH_3), 0.92(3H, *s*, 19-CH_3), 0.95(3H, *d*, J = 6.6 Hz, 21-CH_3), 3.37(1H, *m*, 3β-H), 3.70(3H, *s*, –$COOCH_3$), 3.79(1H, *m*, 7β-H), 5.83(1H, *dt*, J = 15.6 Hz and 1.7 Hz, 25-H), 6.69(1H, *dt*, J = 15.6 Hz and 6.8 Hz, 24-H).

3α,7α-Dihydroxy-25,26-bishomo-5β-cholan-26-oic acid (Scheme 2, **VII***).* Methyl 3α,7α-dihydroxy-27-nor-5β-cholest-24-en-26-oate (Scheme 2, **VI**) (4.6 g) was reduced with H_2 in 50 mL of methanol for 1.5 h using 5% platinum on activated carbon (500 mg) as a catalyst and a hydrogen pressure of 40 psi. After filtration and evaporation, the residue was dissolved in 100 mL of 5% methanolic KOH and refluxed for 1.5 h. The reaction mixture was extracted with ethyl acetate after dilution with water and acidification with 1 N HCl. The residue was crystallized from methanol/ethyl acetate, and colorless crystals of 3α,7α-dihydroxy-25,26-bishomo-5β-cholan-26-oic acid (Scheme 2, **VII**) (3.2 g) were obtained (m.p. = 189–191°C [lit. 191–192°C (19)].

25,26-Bishomo-5β-cholane-3α,7α-26-triol (Scheme 2, **VIII***).* Triethylamine (2.0 mL) and 1.5 mL of ethyl chloroformate were added to a tetrahydrofuran solution (40 mL)



SCHEME 2

of 3α,7α-dihydroxy-25,26-bishomo-5β-cholan-26-oic acid (Scheme 2, **VII**) (2.0 g). This solution was stirred at 0°C for 2 h. Twenty mL of a 10% aqueous solution of sodium borohydride were added, and the reaction mixture was stirred at room temperature for 3 h. The solution was diluted with water, acidified with 1 N HCl and extracted with ethyl acetate. Evaporation gave a residue of 25,26-bishomo-5β-cholane-3α,7α,26-triol (Scheme 2, **VIII**), 1.4 g. ^{1}H NMR 0.69(3H, *s*, 18-CH_3), 0.92(3H, *s*, 19-CH_3), 0.95(3H, *d*, J = 6.6 Hz, 21-CH_3), 3.37(1H, *m*, 3β–H), 3.53(2H, *t*, J = 6.6 Hz, 26-CH_2-OH), 3.79(1H, *m*, 7β-H).

*25,26-Bishomo-26-*p*-toluenesulfoxy-5β-cholane-3α,7α-diol (Scheme 2,* **IX**). To a solution of 25,26-bishomo-5β-cholane-3α,7α,26-triol (Scheme 2, **VIII**) (3.4 g) in tetrahydrofuran (75 mL), there were added 3 mL of triethylamine and 3.4 g of *p*-toluenesulfonyl chloride. This solution was kept at 4°C for 14 d. After dilution with water and acidification with 1 N HCl, the reaction mixture was extracted with ethyl acetate. The residue was purified on a silica gel G 60 column (200 g, 35–70 mesh) using increasing proportions of ethyl acetate in benzene. The fractions containing 25,26-bishomo-26-*p*-toluenesulfoxy-5β-cholane-3α,7α-diol (Scheme 2, **IX**) were collected and evaporated; 1.5 g of (**IX**) was obtained as an oily residue. ^{1}H NMR 0.67(3H, *s*, 18-CH_3), 0.89(3H, *d*, J = 6.6 Hz, 21-CH_3), 0.92(3H, *s*, 19-CH_3), 2.45(3H, *s*, CH_3-phenyl), 3.38(1H, *m*, 3β–H), 3.79(1H, *m*, 7β-H), 4.01(2H, *m*, 26-CH_2-OTs), 7.75 and 7.77(4H, phenyl protons).

25,26-Bishomo-26-iodo-5β-cholane-3α,7α-diol (Scheme 2, **X**). To a solution of 25,26-bishomo-26-*p*-toluenesulfoxy-5β-cholane-3α,7α-diol (Scheme 2, **IX**) (1.5 g) in acetone (15 mL), there were added 15 mL of 10% sodium iodide in acetone. The reaction mixture was allowed to stand at 40°C for 4 h. The solution was diluted with water and extracted with ether. Evaporation gave an oily residue of 25,26-bishomo-26-iodo-5β-cholane-3α,7α-diol (Scheme 2, **X**) (1.4 g). ^{1}H NMR 0.69(3H, *s*, 18-CH_3), 0.93(3H, *s*,19-CH_3), 0-95(3H, *d*, J = 6.6 Hz, 21-CH_3), 3.23(2H, *t*, J = 7.0 Hz, 26-CH_2-I), 3.37(1H, *m*, 3β-H), 3.79(1H, *m*, 7β-H).

Sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate (Scheme 2, **XI**). 25,26-Bishomo-26-iodo-5β-cholane-3α,7α-diol (Scheme 2, **X**) (1.4 g) was dissolved in an ethanolic solution of sodium sulfite (20 mL of ethanol plus 20 mL of 10% aqueous sodium sulfite). The solution was refluxed for 8 h. The ethanol was evaporated, and the residue was dissolved in methanol and filtered. The filtrate was evaporated, and the residue was dissolved in a small amount of water and applied to a 100 g column of Diaion resin (Nippon

Rensui Co., Tokyo, Japan) which is similar to SepPak C_{18}. After washing with 500 mL of water to remove sodium sulfite, the column was eluted with increasing amounts of methanol in water (50 to 80%). The fractions containing sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate (Scheme 2, **XI**) were collected and evaporated. The residue was crystallized from methanol–ethyl acetate, and colorless crystals of (**XI**) (1.1 g) were obtained: m.p. 278–281°C. ^{1}H NMR: 0.67(3H, *s*, 18-CH_3), 0.92(3H, *s*, 19-CH_3), 0.95(3H, *d*, J = 6.4 Hz, 21-CH_3), 2.78(2H, *m*, 26-CH_2-SO_3-Na), 3.36(1H, *m*, 3β-H), 3.79(1H, *m*, 7β-H).

Labeled compounds. [24,25-^{3}H]BishomoCDC-sul (sp. act. 2.6 × 10^6 dpm/mg) was prepared from [24,25-^{3}H]-bishomoCDCA by the method described above for the unlabeled compound. The tritium label was introduced by NEN Research Products by reduction of compound VI with ^{3}H/Pt. The labeled compound was purified to constant specific radioactivity by repeated preparative TLC on silica gel H using the solvent system isooctane/ethyl acetate/acetic acid, 10:10:2, by vol. [24-^{14}C] CDC-tau (sp. act. 9.5 × 10^4 dpm/mg) was synthesized from [24-^{14}C] CDCA (NEN Research Products, Wilmington, DE) by the method of Tserng *et al.* (20). Radiopurity of the labeled compounds was better than 95% as determined by radio-TLC. Each labeled compound was dissolved in 0.9% aqueous NaCl solution for the biological studies.

Liver uptake and intestinal absorption of bishomoCDC-sul. Male golden Syrian hamsters (Sasco, Omaha, NE), weighing 108 ± 4 g, were fed Purina chow (Purina, St. Louis, MO) and water *ad libitum,* and kept under a controlled 12-h light/dark cycle for 1 wk prior to surgery. All animals were operated on between 8:30 and 9:30 a.m. The hamsters were anesthetized by intraabdominal injection of ketamine (Ketaset, Bristol Labs, Syracuse, NY), 20 mg/animal. Injections of ketamine (10 mg/dose) were employed to maintain anesthesia, as required.

A polyethylene catheter (PE-10, 0.28 mm i.d., Clay Adams, Parsippany, NJ) was inserted into the left femoral vein, and saline was infused at a rate of 1.1 mL/h using a Harvard syringe pump (Harvard Apparatus, South Natick, MA). The abdomen was opened by a midline incision, the cystic duct was ligated with a hemostatic clip (Hemo Clip, Edward Weck & Co., Inc., Research Triangle Park, NC), and an external biliary fistula was constructed using PE-10 polyethylene tubing. The urethra was ligated to allow urine to accumulate in the bladder.

In the intravenous infusion study, saline was infused into the femoral vein for 40 min prior to the administration of the [^{3}H]bishomoCDC-sul. The labeled sulfonate was then infused for 20 min at a dose of 50 μg/min (total amount administered, 1 mg); saline was again infused until the end of the experiment. Bile samples were collected in weighed tubes every 20 min for a total period of 4 h.

In the intraduodenal injection experiments, after cannulation of the bile duct and the femoral vein as described above, the labeled sulfonate (0.5 mg) and CDC-tau (0.5 mg) were injected as one bolus into the duodenum. For intraileal injection, the distal end of the ileum was ligated, and the labeled compounds were injected as a bolus into the terminal ileum. Bile samples were collected every 20 min for 4 h. At the end of the experiment, blood and urine were obtained by cardiac puncture and by aspiration from the urinary bladder, respectively, and the liver was excised. All biological specimens were stored at –20°C.

Cholestatic effect of bishomoCDC-sul. In order to estimate the effect of bishomoCDC-sul on bile flow, a dose response experiment was performed as follows: after a 2-h control period, during which saline was infused intravenously, bishomoCDC-sul was administered at increasing rates (0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 μmol/min/kg). These doses were infused for consecutive 40-min periods; bile samples were collected at 20-min intervals.

Analytical techniques. In the metabolic experiments, bile samples were collected every 20 min. Two aliquots were taken from each sample to determine the fate of the administered labeled compounds as follows: (i) The radioactivity of the first aliquot (10 μL) was determined by liquid scintillation counting and (ii) the second aliquot (3 μL) was analyzed by TLC to detect the metabolites of the administered labeled compounds using solvent system CMAW (chloroform/methanol/acetic acid/water, 13:4:2:1). The bile was spotted directly on a TLC plate along with reference compounds. After development (15 cm) and detection of the spots, each TLC plate was cut into segments (1 cm) from the origin to the solvent front, and the radioactivity of each segment was measured as described previously (4).

Aliquots of serum and urine were checked for radioactivity. Bile salts in the liver were analyzed according to the method of Yanagisawa *et al.* (21), and their radioactivity was measured as described above.

Calculation. The numerical data are expressed as mean ± SEM.

RESULTS

Synthesis of bishomoCDC-sul. The synthetic route to bishomoCDC-sul is shown in Scheme 2. BishomoCDCA (Scheme 2, **VII**) was obtained from CDCA (Scheme 2, **I**) *via* the Wittig reaction. After protection of the nuclear hydroxy groups as formyl esters (18), CDCA was reduced to 3α,7α-diformoxy-5β-cholan-24-ol (Scheme 2, **III**) and oxidized to 3α,7α-diformoxy-5β-cholan-24-al (Scheme 2, **IV**) with pyridinium chlorochromate. BishomoCDCA (Scheme, **VII**) was obtained by the reaction of the C_{24}-aldehyde with (carbethoxymethylene)-triphenylphosphorane and hydrogenation of methyl 3α,7α-dihydroxy-27-nor-5β–cholest-24-en-26-oate (Scheme 2, **VI**). BishomoCDCA was treated with ethyl chloroformate in the presence of triethylamine and sodium borohydride to form 25,26-bishomo-5β-cholane-3α,7α,26-triol (Scheme 2, **VIII**). The terminal hydroxy group of the bishomo-cholane triol was selectively converted to the *p*-toluenesulfonyl ester, 25,26-bishomo-26-*p*-toluenesulfoxy-

5β-cholane-3α,7α-diol, by treatment with *p*-toluenesulfonyl chloride in anhydrous tetrahydrofuran solution using triethylamine as a catalytic base. Selective tosylation of the terminal hydroxy group was confirmed by ^{1}H NMR. The signal of the terminal methylene protons was shifted 0.48 ppm downfield by tosylation. The signals of the 18- and 21-methyl protons were shifted upfield by 0.02 and 0.06 ppm, respectively. The tosyl ester (Scheme 2, **IX**) was converted quantitatively into the iodide, 25,26-bishomo-26-iodo-5β-cholane-3α,7α-diol (Scheme 2, **X**). In the C_{26}-iodide derivative (Scheme 2, **X**), the terminal methylene protons were observed at 3.23 ppm. The iodide derivative (Scheme 2, **X**) was refluxed with ethanolic sodium sulfite to give the desired bishomoCDC-sul (Scheme 2, **XI**). The presence of the sulfonic acid at the end of the side chain was confirmed by ^{1}H NMR and ^{13}C NMR (Table 1). The signal of the protons of the terminal methylene was observed at 2.78 ppm, and the signal of the terminal carbon which was bonded directly to the sulfur atom was observed at 52.8 ppm (3,4).

Hepatic uptake and intestinal absorption of bishomoCDC-sul. [^{3}H]-Labeled bishomoCDC-sul was administered intravenously, intraduodenally and intraileally into bile fistula hamsters. The fate of the administered label in bile, liver, blood and urine is summarized in Table 2. Following intravenous administration, 94% of the labeled compound was recovered in bile during the 4-h collection period. In the intraduodenal experiment, only 41% of the label was recovered in bile during the same period. In contrast, after intraileal injection of labeled bishomoCDC-sul, 84% of the radioactivity was recovered in the bile. Radioactivity in blood and urine was below detectable limits.

After intravenous infusion of [^{3}H]-labeled bishomoCDC-sul, the compound appeared rapidly in the bile and more than 60% of the radioactivity was recovered within 1 h. The half time ($t_{1/2}$) of biliary recovery was 45 min.

Biliary secretion of radioactivity after intraduodenal injection of [^{3}H]-labeled bishomoCDC-sul and [^{14}C]-labeled CDC-tau is shown in Figure 1. Both compounds were secreted into the bile more slowly than they were after intravenous administration, and the time course of the recovery of bishomoCDC-sul in the bile closely resembled that of CDC-tau. Figure 2 illustrates the biliary recovery of radioactivity after intraileal injection of the labeled compounds. Both appeared in bile more rapidly than after intraduodenal injection. With CDC-tau, more than 70% of the radioactivity was recovered within 1 h. In contrast, only 40% of labeled bishomoCDC-sul was secreted into the bile during the same period.

TABLE 1
^{13}C Nuclear Magnetic Resonance of Sodium 3α,7α-Dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate[a]

	Compounds[b]			
Carbon	**VIII**	**IX**	**X**	**XI**
1	36.6	36.6	36.6	36.6
2	31.4	31.4	31.4	31.4
3	72.9	72.9	72.9	73.0
4	40.5	40.5	40.5	40.5
5	43.2	43.2	43.2	43.3
6	35.9	35.9	35.9	35.9
7	69.1	69.1	69.1	69.1
8	40.8	40.8	40.8	40.9
9	34.1	34.1	34.1	34.1
10	36.3	36.2	36.2	36.3
11	21.8	21.8	21.8	21.8
12	41.1	41.1	41.1	41.1
13	43.7	43.6	43.6	43.7
14	51.6	51.6	51.6	51.6
15	24.7	24.6	24.6	24.7
16	29.4	29.4	29.4	29.4
17	57.6	57.5	57.5	57.7
18	12.2	12.2	12.2	12.2
19	23.4	23.4	23.4	23.4
20	37.2	37.0	37.1	37.2
21	19.3	19.0	19.2	19.3
22	37.2	36.8	36.9	37.0
23	27.4	26.8	26.1	27.4
24	27.1	26.8	31.9	30.4
25	33.8	29.8	34.8	26.1
26	63.1	72.0	7.1	52.8
Ts				
Phenyl 1		134.7		
2		129.0		
3		131.1		
4		146.4		
Methyl		21.6		

[a]The spectra were measured in CD_3OD solution.
[b]**VIII**, 25,26-bishomo-5β-cholane-3α,7α,26-triol; **IX**, 25,26-bishomo-26-*p*-toluenesulfoxy-5β-cholane-3α,7α-diol; **X**, 25,26-bishomo-26-iodo-5β-cholane-3α,7α-diol; and **XI**, sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate.

TABLE 2
Fate of Radioactivity After Intravenous (iv), Intraduodenal (id) and Intraileal (ii) Administration of [^{3}H]-Labeled BishomoCDC-sul into Bile Fistula Hamsters[a]

Number of animals	Administration	Isotopic recovery (%)				
		Bile	Liver	Blood	Urine	Total
3	iv	94.02 ± 3.46[b]	1.78 ± 1.06	n.d.[c]	n.d.	95.80 ± 4.20
3	id	41.47 ± 3.56	1.32 ± 0.06	n.d.	n.d.	42.79 ± 3.58
3	ii	84.37 ± 1.85	2.81 ± 1.09	n.d.	n.d.	87.18 ± 2.23

[a]The [^{3}H]-labeled bishomoCDC-sul was infused iv into bile fistula hamsters at a rate of 50 μg/min for 20 min. id and ii administration were *via* bolus injection of 0.5 mg. Bile samples were collected for 4 h, and liver, blood and urine samples were then obtained to determine their radioactivity.
[b]The data are expressed as mean ± SEM.
[c]n.d., Not detected.

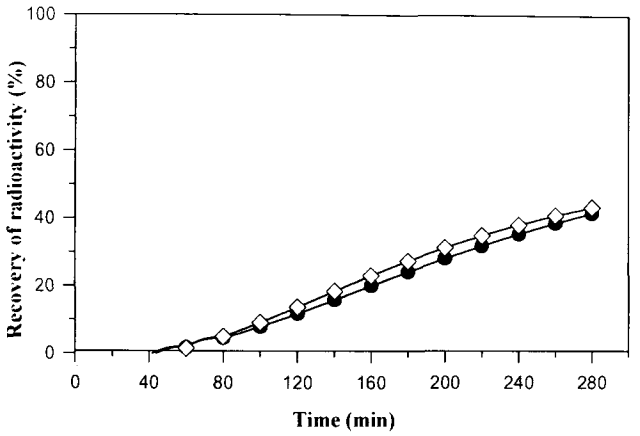


FIG. 1. Cumulative biliary recovery of radioactivity in bile fistula hamsters after intraduodenal injection of [^{3}H]-labeled bishomoCDC-sul and [^{14}C]-labeled chenodeoxycholytaurine. After a 40-min control period, the compounds were injected as a bolus (0.5 mg). The curves represent the means of three hamsters in each group.

From the data of Figure 2, it can be calculated that the $t_{1/2}$s of biliary recovery of CDC-tau and bishomoCDC-sul were 31 and 86 min, respectively.

Figure 3 shows the semi-quantitative analysis by radio-TLC of the bile after intravenous or intraduodenal administration of [^{3}H]labeled bishomoCDC-sul. In these acute experiments, bishomoCDC-sul was secreted into the bile without detectable biotransformation by the liver. The same result was obtained after intraileal administration of bishomoCDC-sul.

Cholestatic effect of bishomoCDC-sul. In order to study the cholestatic properties of bishomoCDC-sul, infusion experiments were performed with the unlabeled compound. Bile flow during intravenus infusion of increasing doses of bishomoCDC-sul is shown in Figure 4. Bile flow remained constant when bishomoCDC-sul was infused at a dose of 0.1

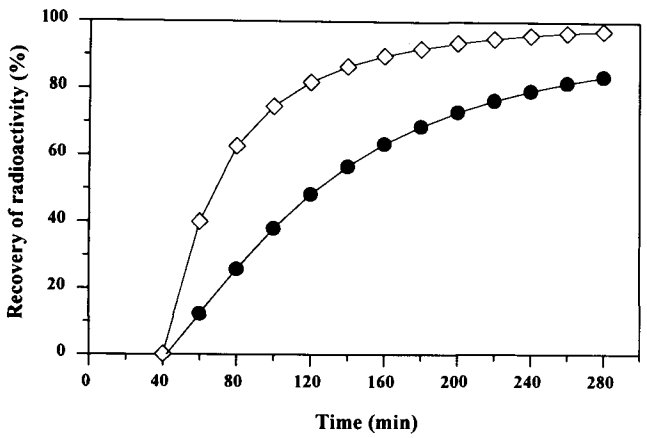


FIG. 2. Cumulative biliary recovery of radioactivity in bile fistula hamsters after intraileal injection of [^{3}H]-labeled bishomoCDC-sul and [^{14}C]-labeled chenodeoxycholytaurine. After a 40-min control period, the compounds were injected as a bolus (0.5 mg). The curves represent the means of three hamsters in each group.

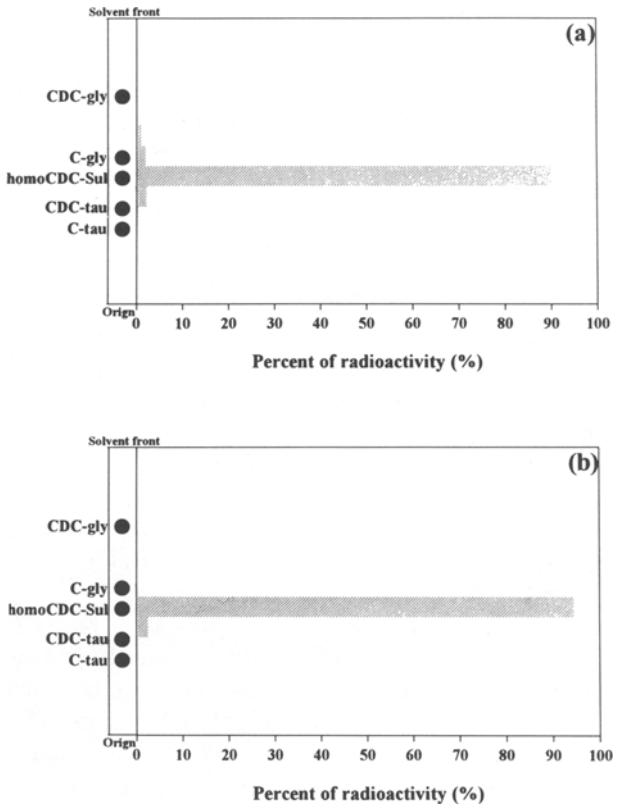


FIG. 3. Thin-layer chromatographic analysis of radioactivity recovered in the bile from bile fistula hamsters after (a) intravenous (iv) and (b) intraduodenal (id) administration of [^{3}H]-labeled bishomoCDC-sul. The bile samples were analyzed by thin-layer chromatography (solvent system CMAW, see Methods section). Reference compounds were as follows: C-tau, cholyltaurine; CDC-tau, chenodeoxycholyltaurine; bishomoCDC-sul, sodium 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate; C-gly, cholylglycine; CDC-gly, chenodeoxycholylglycine. The administered compound was recovered unchanged in the bile. Similar results were obtained following intraileal injection.

to 0.5 μmol/min/kg. An infusion rate of 1 μmol/min/kg of bishomoCDC-sul induced cholestasis.

DISCUSSION

Conjugation of a bile acid with taurine greatly increases its hepatic extraction (22). Bile acid sulfonates are structurally similar to taurine conjugated bile acids and have similar polarities. Therefore, it might be expected that following i.v. infusion bishomoCDC-sul would be extracted by the liver and secreted into the bile, and this was indeed the case. The natural glycine conjugated dihydroxy bile acids are absorbed in the upper small intestine by passive diffusion and by active transport in the terminal ileum. Highly polar taurine conjugates are absorbed poorly by passive diffusion but are transported rapidly by the ileal active transport system (23,24). Figure 1 shows that after intraduodenal injection, bishomoCDC-sul (and CDC-tau) was absorbed relatively slowly, while after intraileal injection the rate of appearance in the bile was much more rapid (Fig. 2). These results indicate that

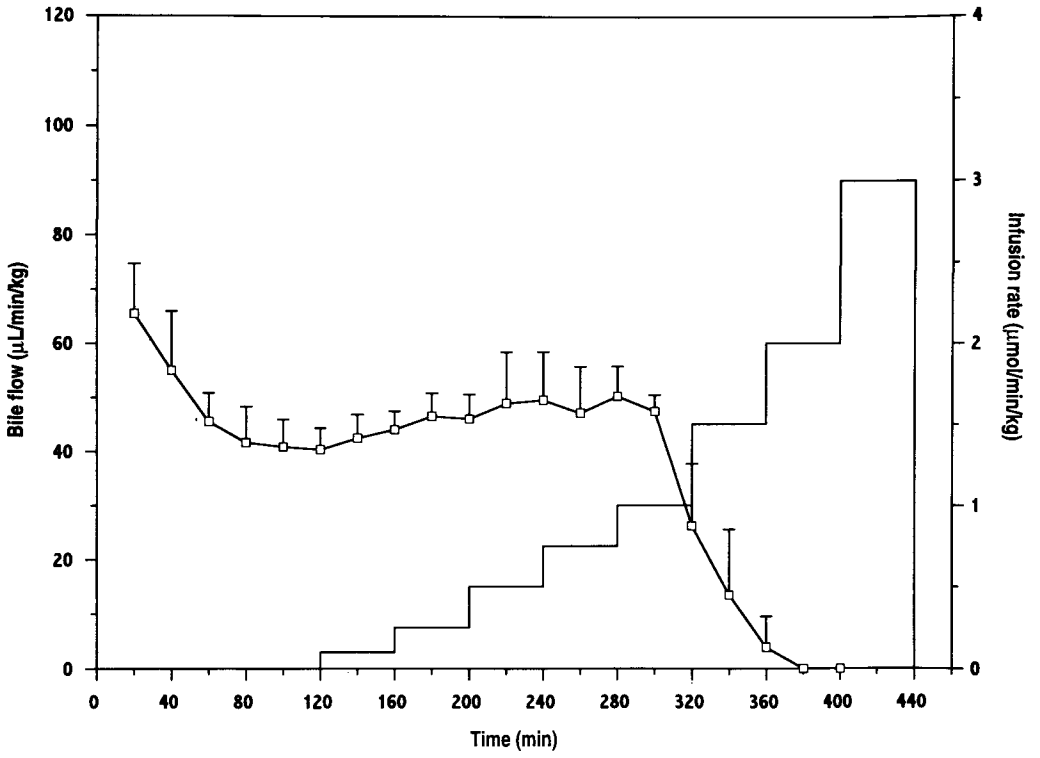


FIG. 4. Effect of bishomoCDC-sul on bile flow in bile fistula hamsters. After a 2-h control period (saline infusion), bishomoCDC-sul was infused intravenously at increasing rates (0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 µmol/min/kg). Each dose was administered for consecutive periods of 40 min, and bile samples were collected every 20 min. (Mean of three hamsters. The vertical lines at each data point represent 1 SEM.)

bishomoCDC-sul is absorbed from the terminal ileum by active transport, just like the natural bile acids, although at a somewhat slower rate. We recently demonstrated that norCDC-sul was absorbed as efficiently as CDC-tau; however, homoCDC-sul was absorbed more slowly than CDC-tau (4). We ascribe these differences to the different polarities of the bile acid sulfonate analogues—by HPLC, the polarity of norCDC-sul was similar to that of CDC-tau, and homoCDC-sul was less polar than CDC-tau. The polarity of bishomoCDC-sul was considerably lower than that of all of the above bile acids, and this may explain, in part, its slower rate of ileal absorption. However, the cholestatic effect of bishomoCDC-sul is related, at least in part, to its relatively slow hepatic clearance. Blood levels of bishomoCDC-sul were not measured during the infusion experiment so that we can only state that the analogue was not present in blood at the end of the infusion period.

In order to define the cholestatic potential of bishomoCDC-sul, we infused the compound at increasing doses. BishomoCDC-sul produced cholestasis at a dose of 1.0 µmol/min/kg. This dose was higher than that of homoCDC-sul (0.75 µmol/min/kg) and lower than that of norCDC-sul (2.0 µmol/min/kg) (4). It is known that hydrophobic bile acids are more hepatotoxic and more cholestatic than hydrophilic bile acids (16,17,24–26). BishomoCDC-sul is more hydrophobic than homoCDC-sul because the former has one more methylene moiety in the side chain. In addition, we demonstrated that CDC-tau was less cholestatic than norCDC-sul even though CDC-tau was more hydrophobic than norCDC-sul (4). These findings suggest that the cholestatic properties of bile acids are a function of their hydrophobicity, the length of the side chain of the bile acid and the presence or absence of a peptide bond in the side chain. BishomoCDC-sul possesses the same side chain length as the glycine conjugated bile acid, CDC-gly. This may explain why bishomoCDC-sul was less cholestatic than homoCDC-sul, even though it was more hydrophobic.

In summary, we synthesized the sulfonate analogue of bishomoCDC, namely bishomoCDC-sul. BishomoCDC-sul was absorbed from the terminal ileum by active transport, efficiently extracted by the liver, and secreted into the bile. In acute experiments, bishomoCDC-sul underwent negligible biotransformation. BishomoCDC-sul was cholestatic in the hamster but to a lesser extent than homoCDC-sul. More studies are needed to decide whether certain sulfonate analogues can serve as long-acting cholelitholytic agents.

ACKNOWLEDGMENTS

Supported in part by USPHS grants R01 DK-43204 from the National Institute of Diabetes and Digestive and Kidney Disease (BIC),

R37 HL-24061 from the National Heart, Lung, and Blood Institute (EHM), and a grant from the Singer-Hellman Fund.

REFERENCES

1. Kihira, K., Yoshii, M., Okamoto, A., Ikawa, S., Ishii, H., and Hoshita, T. (1990) *J. Lipid Res. 31,* 1323–1326.
2. Kihira, K., Okamoto, A., Ikawa, S., Mikami, T., Yoshii, M., Mosbach, E.H., and Hoshita, T. (1991) *J. Biochem. 109,* 879–881.
3. Kihira, K., Mikami, T., Ikawa, S., Okamoto, A., Yoshii, M., Mosbach, E.H., and Hoshita, T. (1992) *Steroids 57,* 193–198.
4. Miki, S., Mosbach, E.H., Cohen, B.I., Yoshii, M., Ayyad, N., and McSherry, C.K. (1992) *J. Lipid Res. 33,* 1629–1637.
5. Mikami, T., Kihira, K., Ikawa, S., Yoshii, M., Miki, S., Mosbach, E.H., and Hoshita, T. (1993) *J. Lipid Res. 34,* 429–435.
6. Cohen, B.I., Miki, S., Mosbach, E.H., Ayyad, N., Stenger, R.J., Mikami, T., Yoshii, M., Kihira, K., and Hoshita, T. (1993) *Hepatology 17,* 103–110.
7. Danzinger, R.G., Hofmann, A.F., Schoenfield, L.J., and Thistle, J.L. (1972) *N. Engl. J. Med. 286,* 1–8.
8. Makino, I., Shinozaki, K., Yoshino, K., and Nagasawa, S. (1975) *Jpn. J. Gastroenterol. 72,* 690–702.
9. Roehrkasse, R., Fromm, H., Malavolti, M., Tunuguntla, A.K., and Ceryak, S. (1986) *Dig. Dis. Sci. 31,* 1032–1040.
10. Bazzoli, F., Fromm, H., Sarva, R.P., Sembrat, R.F., and Ceryak, S. (1982) *Gastroenterology 83,* 753–760.
11. Fedorowski, T., Salen, G., Colallilo, A., Tint, G.S., Mosbach, E.H., and Hall, J.C. (1977) *Gastroenterology 77,* 1131–1137.
12. Palmer, R.H. (1972). *Arch. Intern. Med. 130,* 606–617.
13. Miyai, K., Javitt, N.B., Gochman, N., Jones, H.M., and Baker, D. (1982) *Lab. Invest. 46,* 428–437.
14. Reddy, B.S., and Watanabe, K. (1979) *Cancer Res. 39,* 1521–1524.
15. Turjman, N., and Nair, P.P. (1981) *Cancer Res. 41,* 3761–3763.
16. Drew, R., and Priestly, B.G. (1978) *Experientia 35,* 809–811.
17. Heuman, D.M., Mills, A.S., McCall, J., Hylemon, P.B., Pandak, W.M., and Vlahcevic, Z.R. (1991) *Gastroenteroloqy 100,* 203–211.
18. Schteingart, C.D., and Hofmann, A.F. (1988) *J. Lipid Res. 29,* 1387–1395.
19. Kihira, K., Batta, A.K., Mosbach, E.H., and Salen, G. (1979) *J. Lipid Res. 20,* 421–427.
20. Tserng, K-Y., Hachey, D.L., and Klein, P.D. (1977) *J. Lipid Res. 19,* 925–928.
21. Yanagisawa, J., Itoh, M., Ishibashi, M., Miyazaki, H., and Nakayama, F. (1980) *Anal. Biochem. 104,* 75–86.
22. Carey, M.C., and Cahalane, M.J. (1988) in *The Liver: Biology and Pathology*, 2nd Edn. (Arias, I.M., Jacoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds.), pp. 587–588, Raven Press, New York.
23. Dietschy, J.M. (1968) *J. Lipid Res. 9,* 297–309.
24. Schiff, E.R., Small, N.C., and Dietschy, J.M. (1972) *J. Lipid Res. 51,* 1351–1362.
25. Attili, A.F., Angelico, M., Cantafora, A., Alvaro, D., and Capocacca, L. (1986) *Med. Hypotheses 19,* 57–69.
26. Heuman, D.M., Pandak, W.M., Hylemon, P.B., and Vlahcevic, Z.R., *Hepatology 14*, 920–926.

[Received July 18, 1994, and in revised form November 17, 1994; Revision accepted December 7, 1994]

Chemical and Enzymatic Preparation of Acylglycerols Containing C_{18} Furanoid Fatty Acids

Marcel S.F. Lie Ken Jie* and M.S.K. Syed-Rahmatullah

Department of Chemistry, The University of Hong Kong, Hong Kong

ABSTRACT: C_{18} furanoid triacylglycerol [glycerol tri-(9,12-epoxy-9,11-octadecadienoate)] was prepared by chemical transformation of triricinolein isolated from castor oil. The procedure involved oxidation, epoxidation and cyclization of the epoxy-keto intermediate with sodium azide and ammonium chloride in aqueous ethanol. The furanoid triacylglycerol was also obtained by esterification of C_{18} furanoid fatty acid with glycerol using Novozyme 435 (Novo Nordisk A.S., Bagsvaerd, Denmark) as biocatalyst. When Lipozyme (Novo Nordisk A.S.) was used, a mixture of the furanoid 1(3)-*rac*-monoacylglycerol and 1,3-diacylglycerol was obtained. In order to obtain the C_{18} furanoid 1,2(2,3)-diacylglycerol, selective hydrolysis of the furanoid triacylglycerol was achieved using porcine pancreatic lipase in *tris*(hydroxymethyl) methylamine buffer. Interesterification of triolein with methyl C_{18} furanoid ester in the presence of Lipozyme showed maximum incorporation of 34% of furanoid fatty acid. Extension of the interesterification to vegetable oils (olive, peanut, sunflower, corn and palm oil) allowed a maximum of 24% furanoid acid incorporation to be achieved.
Lipids 30, 79–84 (1995).

A considerable number of long-chain fatty acids containing a furan ring system as part of the alkyl chain have been isolated from a seed oil (1), the latex of the rubber plant (2,3), algal species (4) and from many other plant tissues (5). Such furanoid fatty acids are also found in the liver of some freshwater and marine fishes (6–12), in the crayfish (13–15), and in sponges (16) and soft corals (17). Furanoid fatty acids have also been isolated from the lipid extracts of higher animals (18), including human (19). Furanoid fatty acids are found acylated to glycerol as part of the triacylglycerol pool in seed oils (2,3) and in fish liver (12), but are esterified to cholesterol in the testes of fish (12). The role and function of these unusual fatty acids remain to be determined. In our continuing effort to study the properties of furanoid fatty acids and their derivatives, we describe the synthesis of C_{18} furanoid acylglycerols (mono-, di- and triacylglycerols) by partial chemical synthesis and by the incorporation of a C_{18} furanoid fatty acid into triolein and other vegetable oils by enzymatic esterification and interesterification methods.

*To whom correspondence should be addressed at Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong.

Abbreviations: IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy.

MATERIALS AND METHODS

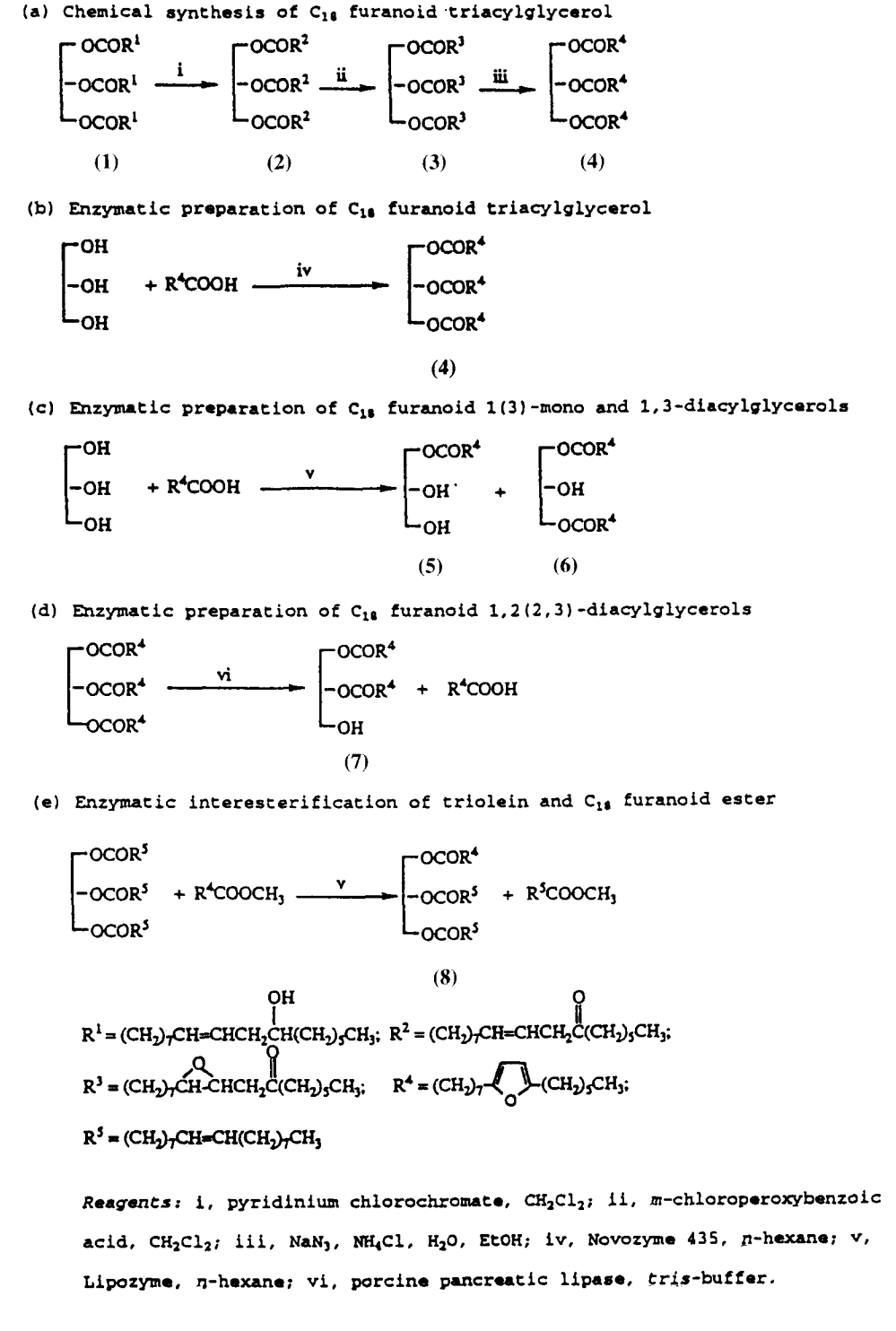
Castor, palm, olive, peanut, corn and sunflower oil were purchased from local supermarkets. All solvents were distilled before use. Petroleum ether refers to the fraction with b.p. of 40–60°C. Triolein was synthesized by enzymatic (Novozyme 435) esterification of glycerol with oleic acid. Pyridinium chlorochromate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Lipozyme [lipase isolated from *Rhizomucor miehei* and immobilized on anion exchange resin, activity 24 BIU (BIU—Batch Interesterification Unit, *viz.* 1 μmol of palmitic acid incorporated into triolein per min under standard conditions as defined by the manufacturer)] and Novozyme 435 (lipase isolated from *Candida antarctica* and immobilized on macroporous acrylic resin, activity 40 BIU/g are gifts from Novo Nordisk A.S. (Bagsvaerd, Denmark). Porcine pancreatic lipase (type II, 179 units/mg protein) was purchased from Sigma (St. Louis, MO). Thin-layer, column and gas–liquid chromatographic analyses were performed as described elsewhere (20). Melting points were determined on a hot stage microscope apparatus and values are uncorrected. Methyl 9,12-epoxy-9,11-octadecadienoate was prepared by a previously described method (21). Infrared, ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded as described elsewhere (20).

Triricinolein [glycerol tri-(12-hydroxy-9-*cis*-octadecenoate)] was obtained from castor oil as follows: castor oil (10.0 g) was dissolved in a mixture of petroleum ether/diethyl ether (5:2, vol/vol) (100 mL). The solution was passed through a silica (100 g) column. The column was eluted with a mixture of petroleum ether/diethyl ether (7:3, vol/vol; 200 mL) to remove triacylglycerols containing two ricinoleic acids and a nonhydroxylated fatty acid. The column was further eluted with a mixture of petroleum ether/diethyl ether (2:3, vol/vol; 400 mL), which furnished pure triricinolein (7.0 g) on evaporation of the solvent.

Copyright © 1995 by AOCS Press

EXPERIMENTAL PROCEDURES

Chemical synthesis of C_{18} furanoid triacylglycerol by the oxidation of triricinolein (**1**) *(Scheme 1a).* A solution of pyridinium chlorochromate (2.0 g, 9.3 mmol) in dichloromethane (50 mL) was added to triricinolein (**1**, 2.0 g, 2.15 mmol) and stirred for 4 h at room temperature. Diethyl ether (100 mL) was added to precipitate the chromium salts, and the reaction mixture was filtered through a column of Florisil (50 g). The adsorbed reaction product was further eluted with diethyl ether (200 mL). The solvent of the eluate was evaporated to give a residue (1.9 g), which was purified by silica (30 g) col-



SCHEME 1

umn chromatography using a mixture of petroleum ether/diethyl ether (7:3, vol/vol; 150 mL) as the eluent to give pure glycerol tri-(12-oxo-9-*cis*-octadecenoate) (**2**, 1.7 g, 85%) as a viscous liquid. Infrared (cm^{-1}) film: 3010, 1740(C=O, ester), 1715(C=O, oxo). ^1H NMR ($CDCl_3$, δ) 0.84(*t*, 9H, CH_3), 1.2–1.5(*m*, 48H, CH_2), 1.5–1.9(*m*, 12H, CH_2), 1.9–2.0(*m*, 6H, CH_2-CH=CH), 2.3(*t*, 6H, 2-*H*), 2.44(*t*, 6H, 13-*H*), 3.16(*d*, 6H, 11-*H*), 4.24(*m*, 4H, glyceryl CH_2), 5.3(*m*, 1H, glyceryl C*H*), 5.6(*m*, 6H, C*H*=C*H*). ^{13}C NMR ($CDCl_3$, ppm) 13.98(C-18), 22.5(C-17), 23.84(C-14), 24.87(C-3), 27.52(C-8), 28.93, 29.09, 29.31, 31.64(C-16), 34.02(C-2 of 1-acyl, 2C), 34.18(C-2 of 2-acyl, 1C), 41.71(C-11), 42.36(C-13), 62.14(glyceryl, CH_2), 69.02(glyceryl, *C*H), 121.13(C-10), 133.49(C-9), 172.76(C-1 of 2-acyl, 1C), 173.14(C-1 of 1-acyl, 2C) and 209.01(C-12, C=O oxo).

*Epoxidation of glycerol tri-(12-oxo-9-*cis*-octadecenoate)* (**2**). A mixture of the keto-ene triglyceride intermediate (**2**, 1.0 g, 1.1 mmol), dichloromethane (25 mL and *m*-chloroperoxybenzoic acid (1.2 g, 50% purity, 3.5 mmol) was stirred for 8 h at room temperature. Aqueous sodium sulfite (10%, 20 mL) was added and the organic phase was isolated. The organic phase was successively washed with aqueous $NaHCO_3$ (5%, 15 mL), water (2 x 20 mL), and dried (Na_2SO_4). Evaporation of the filtrate furnished crude glycerol tri-(9,10-epoxy-12-oxo-octadecanoate) (**3**, 0.9 g, 82%), which was purified by recrystallization from a mixture of petroleum ether/diethyl ether (1:1, vol/vol) to yield pure glycerol tri-(9,12-epoxy-12-oxo-octadecanoate) (**3**, 0.7 g, 73%). m.p. 73°C; IR (cm^{-1}) film 1740(C=O, ester), 1700(C=O, oxo), 842(C-H, epoxy ring). ^1H NMR ($CDCl_3$, δ) 0.88(*t*, 9H, CH_3), 1.2–1.8(*m*, 60H, CH_2), 2.2–2.45(*m*, 12H, 2-*H*, 13-*H*), 2.64(*d*, 6H, 11-*H*), 2.95(*m*, 3H, epoxy C-*H*, 9-*H*), 3.3(*m*, 3H, epoxy C-*H*, 10-*H*), 4.0–4.4(*m*, 4H, glyceryl CH_2), 5.24(*m*, 1H, glyceryl C*H*). ^{13}C NMR ($CDCl_3$, ppm) 14.03(C-18), 22.48(C-17), 23.62(C-14), 24.81(C-3), 26.49(C-8), 28.01(C-7), 28.29, 28.98, 29.20, 29.31, 31.58(C-16), 34.02(C-2 of 1-acyl, 2C), 34.18(C-2 of 2-acyl, 1C), 41.71(C-11), 43.39(C-13), 52.39(C-10), 56.50(C-9), 62.14(glyceryl CH_2), 69.12(glyceryl *C*H), 172.82(C-1 of 2-acyl, 1C), 173.20(C-1 of 1-acyl, 2C), 208.41(C-12, C=O oxo, 3C).

Cyclization of epoxy-keto triglyceride (**3**). A mixture of compound **3** (0.5 g, 0.5 mmol.), sodium azide (0.9 g, 14 mmol), ammonium chloride (0.75 g, 14 mmol), water (3.0 mL) and ethanol (20 mL) was refluxed for 40 min. The reaction mixture was cooled and extracted with diethyl ether (2 x 50 mL). The ethereal extract was washed with water (2 x 20 mL) and dried (Na_2SO_4). Silica column chromatographic purification using a mixture of petroleum ether/diethyl ether (9:1, vol/vol) as eluent gave pure glycerol tri-(9,12-epoxy-9,11-octadecadienoate) (**4**, 0.3 g, 66%) as a viscous liquid. IR (cm^{-1}) film 3100(C-H furan), 1740(C=O ester), 1610, 1560, 1010(C-O-C furan), 780. ^1H NMR ($CDCl_3$, δ) 0.88(*t*, 9H, CH_3), 1.2–1.4(*m*, 36H, CH_2), 1.6–1.8(*m*, 24H, CH_2), 2.3(*t*, 6H, 2-*H*), 2.55(*t*, 12H, 8-*H*, 13-*H*), 4.1–4.4(*m*, 4H, glyceryl CH_2), 5.26(*m*, 1H, glyceryl C*H*) 5.83(*s*, 6H, furan C*H*). ^{13}C NMR ($CDCl_3$, ppm) 14.09(C-18), 22.60(C-17), 24.83(C-3, 1-acyl), 24.86(C-3, 2-acyl), 28.04, 28.09, 28.91, 29.03, 31.61(C-16), 34.01(C-2 of 1-acyl, 2C), 34.18(C-2 of 2-acyl, 1C), 62.11(glyceryl CH_2), 68.90(glyceryl *C*H), 104.81(C-10), 104.87(C-11), 154.43(C-9), 154.69(C-12), 172.85(C-1 of 2-acyl, 1C), 173.26(C-1 of 1-acyl, 2C).

Enzymatic method: enzymatic esterification of glycerol with furanoid fatty acid (9,12-epoxy-9,11-octadecadienoic acid) in the presence of Novozyme 435 (Scheme 1b). A mixture of 9,12-epoxy-9,11-octadecadienoic acid (0.3 g, 1.0 mmol), glycerol (53 mg, 0.06 mmol), Novozyme 435 (35 mg) and *n*-hexane (2 mL) was stirred at 60°C for 2 h. Molecular sieve (4 Å, 0.5 g) was added (to remove co-produced water), and the mixture was stirred for a further 6 h. The mixture was centrifuged to separate the molecular sieve and enzyme from the reaction mixture. The supernatant liquid was isolated and silica column chromatographic separation by gradient elution using mixture of petroleum ether/diethyl ether (9:1 to 3:2, vol/vol) as eluent gave: glycerol tri-(9,12-epoxy-9,11-octadecadienoate) (**4**, 0.16 g, 47%) and glycerol 1,3-di-(9,12-epoxy-9,11-octadecadienoate) (0.06 g, 10%).

*Preparation of C_{18} furanoid 1(3)-*rac*-monoacylglycerol* (**5**) *and 1,3-diacylglycerols* (**6**) *(Scheme 1c): Enzymatic esterification of glycerol with 9,12-epoxy-9,11-octadecadienoic acid in the presence of Lipozyme.* A mixture of methyl 9,12-epoxy-9,11-octadecadienoic acid (0.4 g, 1.3 mmol), glycerol (60 mg, 0.6 mmol), Lipozyme (46 mg), silica gel (TLC grade) (60 mg) and *n*-hexane (4 mL) was stirred at 60°C for 2 h. Molecular sieve (0.5 g) was added and the reaction mixture stirred for a further 10 h. Isolation and silica column chromatographic separation by gradient elution with mixtures of petroleum ether/diethyl ether (9:1 to 7:3, vol/vol) as eluent yielded: (a) glycerol 1(3)-*rac*-(9,12-epoxy-9,11-octadecadienoate) (**5**, 70 mg, 20%). ^1H NMR ($CDCl_3$, δ) 0.88(*t*, 3H, CH_3), 1.2–1.5(*m*, 12H, CH_2), 1.6–1.8(*m*, 6H, CH_2), 2.3(*t*, 2H, 2-*H*), 2.55(*t*, 4H, 8-*H*, 13-*H*), 3.0(*s*, 2H, O-*H*), 3.65(*d*, glyceryl CH_2-OH), 3.93(*m*, glyceryl C*H*), 4.19(*d*, 2H, glyceryl CH_2), 5.83(*s*, 2H, furan C*H*). ^{13}C NMR ($CDCl_3$, ppm) 14.09(C-18), 22.59(C-17), 24.92(C-3), 28.12(C-7, C-8, C-13, C-14, 4C), 28.98, 31.64(C-16), 34.18(C-2), 63.49(glyceryl CH_2-OH), 65.23(glyceryl CH_2-O-CO-), 70.37(glyceryl, *C*H-OH), 104.94(C-10, C-11, furan), 154.45(C-9, C-12, furan) and 174.28(C-1); (b) glycerol 1,3-di(9,12-epoxy-9,11-octadecadienoate) (**6**, 150 mg, 45%). ^1H NMR ($CDCl_3$, δ) 0.88(*t*, 6H, CH_3), 1.1–1.5(*m*, 24H, CH_2), 1.6–1.8(*m*, 12H, CH_2), 2.0(*s*, 2H, O-H), 2.3(*t*, 4H, 2-*H*), 2.55(*t*, 8H, 8-*H*, 13-*H*), 4.1–4.15(*m*, 5H, glyceryl C*H* and CH_2), 5.8(*s*, 4H, furan C*H*). ^{13}C NMR ($CDCl_3$, ppm) 14.09(C-18), 22.63(C-17), 24.84(C-3), 28.02(C-8/C-13), 28.08(C-8/C-13). 28.90, 29.11, 29.17, 29.34, 29.70, 31.61(C-16), 34.07(C-2), 65.05, 68.33, 104.81, 104.88(furan, *C*-H), 154.42, 154.68(furan, *C*) and 173.87(C-1); and (c) glycerol tri-(9,12-epoxy-9,11-octadecadienoate) (**4**, 17 mg, 5%).

*Preparation of glycerol 1,2(2,3)-*rac*-di-(9,12-epoxy-9,11-octadecadienoates by pancreatic lipase hydrolysis of glycerol tri-(9,12-epoxy-9,11-octadecadienoate)* (**4**) *(Scheme 1d).* Glycerol tri(9,12-epoxy-9,11-octadecadienoate) (**4**, 0.2 g)

was suspended in *tris*(hydroxymethyl) methylamine buffer (2 mL, 1M, pH 8.0) in a screw-capped test tube. Aqueous calcium chloride (0.4 mL, 22 g in 100 mL) and sodium cholate (1 mL, 0.1% solution) were added and the mixture kept at 40°C for 5 min. A suspension of porcine pancreatic lipase (40 mg) in *tris*(hydroxymethyl) methylamine buffer (1 mL) at 40°C was added, and the mixture was stirred at 40°C for 10 min. Dilute HCl (6M, 1.0 mL) and diethyl ether (2 mL) were added, and the mixture was centrifuged. The ethereal layer was isolated and the aqueous solution was re-extracted with diethyl ether (2 x 2 mL). The combined ethereal extract was dried (Na_2SO_4) and the filtrate was evaporated under a stream of nitrogen. The residue was quickly separated on a silica (10 g) column using a mixture of petroleum ether/diethyl ether (1:1 to 0:1, vol/vol) as the eluent by gradient elution to give glycerol 1,2(2,3)-*rac*-di-(9,12-epoxy-9,11-octadecadienoate) (**7**, 80 mg, 35%). ^{1}H NMR ($CDCl_3$, δ) 0.88(*t*, 6H, CH_3), 1.2–1.5(*m*, 24H, CH_2), 1.55–1.8(*m*, 17H, CH_2 and O-*H*), 2.30(*t*, 4H, 2-*H*), 2.6(*t*, 8H, 8-*H* and 13-*H*), 3.70 (*d*, 2H, glyceryl CH_2-OH), 4.3(*m*, 2H, glyceryl CH_2), 5.10(*m*, 1H, glyceryl C*H*) and 5.82(*s*, 4H, furan C*H*). ^{13}C NMR ($CDCl_3$, ppm) 14.03(C-18), 22.59(C-17), 24.76 and 24.86(C-3), 28.12, 28.60, 29.04, 31.64(C-16), 34.08 and 34.29(C-2), 61.49(glyceryl CH_2OH), 62.19(glyceryl CH_2), 72.1(glyceryl C*H*), 104.90(furan C*H*), 154.45(C-9), 154.72(C-12), 173.30 and 173.68(C-1).

Incorporation of 9,12-epoxy-9,11-octadecadienoic acid into triolein by enzymatic interesterification using Lipozyme (Scheme 1d). A mixture of triolein (0.2 g, 0.23 mmol), methyl 9,12-epoxy-9,11-octadecadienoate (0.2 g, 0.68 mmol), Lipozyme (40 mg) and *n*-hexane (2 mL) was stirred at 60°C for 4 h. The mixture was centrifuged, and the supernatant liquid was eluted through a silica column using a mixture of petroleum ether/diethyl ether (4:1, vol/vol) as the eluent to give glycerol 1(3)-*rac*-(9,12-epoxy-9,11-octadecadienoate)-2,3(1,2)-dioleate (**8**, 0.12 g, 60%). IR (cm^{-1}) film 3100(C-H) furan), 1740(C=O, ester), 1610, 1560 and 1010 (C-O-C, furan). ^{1}H NMR ($CDCl_3$, δ): 0.84(*t*, 9H, CH_3), 1.2–1.5(*m*, CH_2), 2.0(*m*, 8H, CH_2CH=CH), 2.30(*t*, 6H, 2-*H*), 2.50(*t*, 4H, CH_2-furan), 4.2(*m*, 4H, glyceryl CH_2), 5.36(*m*, 1H, glyceryl C*H*) and 5.74(*s*, 2H, furan C*H*). ^{13}C NMR ($CDCl_3$, ppm) 14.08(C-18, furanoyl), 14.12(C-18, oleoyl), 22.59(C-17, furanoyl), 22.70(C-17, oleoyl), 24.83(C-3, furanoyl), 24.86(C-3, 1-oleoyl), 24.90(C-3, 2-oleoyl), 27.19, 27.22, 27.24, 28.04, 28.09, 28.11, 28.91, 29.03, 29.07, 29.11, 29.13, 29.15, 29.21, 29.33, 29.34, 29.54, 29.67, 29.73, 29.78, 31.61(C-16, furanoyl), 31.92(C-16, oleoyl), 34.02(C-2, furanoyl), 34.05(C-2, 1-oleoyl), 34.21(C-2, 2-oleoyl), 62.18(glyceryl CH_2), 68.90(glyceryl C*H*), 104.80(C-10, furanoyl), 104.86(C-11, furanoyl), 129.69(C-9, 2-oleoyl), 129.72(C-9, 1-oleoyl), 130.03(C-10, 1-oleoyl), 130.04(C-10, 2-oleoyl), 154.43(C-9, furanoyl), 154.69(C-12, furanoyl), 172.86(C-1, 2-oleoyl), 173.24(C-1, furanoyl) and 173.27(C-1, 1-oleoyl).

The same interesterification procedure was repeated using different vegetable oils (olive, peanut, sunflower, corn and palm oil). In each case the triacylglycerol fraction was isolated by silica column chromatography. The extent of the incorporation of the furanoid fatty acid into the various seed oils was reflected by the amount of furanoid fatty acid present in the total fatty acid composition of the interesterified seed oils as given in Table 1.

RESULTS AND DISCUSSION

To synthesize C_{18} furanoid triacylglycerol (**4**), two approaches were investigated (Scheme 1, a and b). Starting from triricinolein (**1**) isolated from castor oil, oxidation of the hydroxy function in high yield was achieved by using commercially available pyridinium chlorochromate in CH_2Cl_2. This method was preferred to the low-yield two-phase (diethyl ether/water) chromic acid procedure often employed in the synthesis of the furanoid fatty ester from methyl ricinoleate (22), which caused overoxidation of the substrate. Subsequent epoxidation of the keto-ene triacylglycerol (**2**) was successfully accomplished with *m*-chloroperoxybenzoic acid. Using the high yield and efficient NaN_3/NH_4Cl/EtOH method de-

TABLE 1
Fatty Acid Composition of Triacylglycerols of Selected Vegetable Seed Oils Before and After Interesterification Reaction with a C_{18} Furanoid Fatty Acid Methyl Ester Using Lipozyme at 60°C

	Palm		Olive		Peanut		Corn		Sunflower	
Fatty acid	BI[a]	AI[a]	BI	AI	BI	AI	BI	AI	BI	AI
					(wt%)					
16:0	42.5	25.8	10.7	8.0	9.8	6.6	9.3	5.7	6.0	4.1
18:0	3.4	1.6	1.5	1.4	2.3	1.5	2.6	1.7	3.3	2.3
18:1	38.3	33.8	60.1	60.1	41.8	34.2	28.3	21.2	24.6	18.5
18:2	9.5	9.7	19.5	7.5	30.6	28.6	54.6	43.3	60.7	47.5
18:F[b]	0	23.5	0	15.3	0	22.4	0	22.2	0	24.3
20:0	—	—	—	—	1.2	0.8	—	—	—	—
20:1	—	—	—	—	1.7	0	—	—	—	—
22:0	—	—	—	—	3.0	1.8	—	—	—	—
24:0	—	—	—	—	2.7	0.9	—	—	—	—

[a]BI, before interesterification; AI, after interesterification. All numbers in wt%.
[b]18:F, 9,12-epoxy-9,11-octadecadienoic acid.

veloped by us (21), the keto-epoxy triacylglycerol intermediate was cyclized to the requisite C_{18} furanoid triacylglycerol (**4**) in good yield (66%).

Novozyme 435 and Lipozyme are reported to be efficient biocatalysts for esterification and interesterification, respectively (23,24). To obtain the same C_{18} furanoid triacylglycerol (**4**) by enzymatic (biocatalytic) means, the C_{18} furanoid fatty acid was first prepared by well-established methods (21). Enzymatic esterification of C_{18} furanoid fatty acid to glycerol was conducted using Novozyme 435 (Scheme 1b) or Lipozyme at 60°C in *n*-hexane (Scheme 1c). Molecular sieve was added to the reaction mixture during incubation to remove the water liberated during the process and to direct the course of reaction toward esterification. When Novozyme 435 was used as the biocatalyst, the C_{18} furanoid triacylglycerol (**4**) was obtained in 47% yield together with 1,3-diacylglycerol (10%). The C_{18} furanoid triacylglycerol (**4**) obtained by both chemical transformation of triricinolein or by enzymatic esterification of glycerol with C_{18} furanoid fatty acid was characterized by the signals at δ 4.1–4.4(*m*), 5.26(*m*) for the glyceryl protons and 5.83(*s*) for the furan protons in the ^{1}H NMR spectrum. These assignments were based on the intensities of the signals and from the reported chemical shift values of methyl C_{18} furanoid fatty ester (22). The signals at 62.11 and 68.90 for the glyceryl carbon shifts, and 104.81, 104.87, 154.43 and 154.69 ppm for the furan carbon shifts in the ^{13}C NMR spectrum confirmed the structure of compound **4**.

When Novozyme 435 was replaced by Lipozyme, the esterification process was poor in producing triacylglycerol. Instead the reaction furnished mainly the 1(3)-*rac*-(9,12-epoxy-9,11-octadecadienoate) (**5**, 20%) and glycerol 1,3-di(9,12-epoxy-9,11-octadecadienoate) (**6**, 45%), due to its specific ability to esterify fatty acids to the 1- and 3-positions of the glycerol molecule. Only a small amount of the triacylglycerol (**4**, 5%) was produced (Scheme 1c). From these results, Novozyme 435 is the choice biocatalyst to be used for esterification of fatty acids to glycerols to yield triacylglycerols, whereas Lipozyme is best used for obtaining 1(3)-*rac*-mono- and 1,3-diacylglycerols.

The C_{18} furanoid 1,2(2,3)-*rac*-diacylglycerol (**7**) was also prepared by selectively hydrolyzing the 1(3)-position of the corresponding C_{18} furanoid triacylglycerol (**4**) with porcine pancreatic lipase according to the IUPAC method (25) with minor modification (Scheme 1d). Rapid silica column chromatographic separation of the product furnished unreacted C_{18} furanoid triacylglycerol (**4**, 10%) and glycerol 1,2(2,3)-*rac*-di(9,12-epoxy-9,11-octadecadienoate) (**7**, 35%). However, the latter compound isomerized readily to the 1,3-diacylglycerol on standing at room temperature.

The incorporation of C_{18} furanoid fatty ester to triacylglycerols was investigated by the interesterification reaction with triolein (Scheme 1e) and some vegetable oils. Lipozyme was selected as the biocatalyst for this purpose. The experimental conditions were first optimized by using triolein as a model substrate. The reaction temperature was kept constant at 60°C for 4 h during each trial. The amount of furanoid fatty acid incorporation into the triolein at the 1- and 3-positions of the glycerol backbone depended on the ratio of the amount of the triolein to furanoid fatty ester used. When a 1:1 ratio of triolein and furanoid fatty ester was used, only 14.5% of furanoid fatty acid was incorporated. By changing the ratio of triolein and furanoid fatty ester to 1:3, a 34% incorporation of furanoid fatty acid into the triolein molecule was observed. But when a ratio of 1:9 of triolein to furanoid ester was tried, the rate of interesterification was not improved as the incorporation of the furanoid fatty acid remained at a constant level of 34%. By extending the reaction time from 4 to 8 h, increased amounts of diacylglycerols, monoacylglycerols and free fatty acids were obtained, but the amount of furanoid acid incorporation decreased with time.

From the interesterification study of vegetable oils (olive, peanut, sunflower, corn and palm oil), it became apparent that the incorporation of the C_{18} furanoid fatty ester into the various natural triacylglycerols could only be achieved to a maximum of 24.3% (Table 1). Increasing the incubation period of the biocatalyzed interesterification process did not improve the degree of incorporation of the C_{18} furanoid ester into the triacylglycerols of the vegetable oils.

The ^{13}C NMR spectral results of the interesterified triacylglycerol product of triolein showed that the C_{18} furanoid fatty acid was esterified to the 1(3)-position of the glycerol backbone only. This was deduced by referring to the ^{13}C NMR result obtained for glycerol tri-(9,12-epoxy-9,11-octadecadienoate). The carbon shifts of the olefinic carbon atoms of oleic acid moieties in this product also showed that the oleic acid occupied both the 1- and 2-positions of the triacylglycerol molecule from the signals at: 129.72(C-9, 1-acyl), 129.69(C-9, 2-acyl), 130.03(C-10, 1-acyl) and 130.04(C-10, 2-acyl). Signals associated with the furan nucleus appeared at 104.80, 104.86, 154.43 and 154.69 ppm. It can be concluded from this study that enzymatic interesterification using Lipozyme allowed only the 1-acyl chain of the triacylglycerol to be replaced by the C_{18} furanoid ester.

This study confirms that Lipozyme, Novozyme and porcine pancreatic lipase do not discriminate the synthetic C_{18} furanoid fatty acid over other common natural fatty acids in the process of esterification, interesterification or hydrolysis involving such biocatalysts.

ACKNOWLEDGMENTS

The authors thank the Lipid Research Fund, the Research Grant Council and the Research Grants Committee of the University of Hong Kong for financial assistance, and Novo Nordisk A/S (Bagsvaerd, Denmark) for samples of lipases.

REFERENCES

1. Morris, L.J., Marshall, M.O., and Kelly, W. (1966) *Tetrahedron Lett.*, 4249–4253.
2. Hasma, H., and Subramaniam, A. (1978) *Lipids 13*, 905–907.
3. Lie Ken Jie, M.S.F., and Sinha, S. (1981) *Phytochemistry 20*. 1863–1866.

4. Batna, A., Scheinkonig, J., and Spiteller, G. (1993) *Biochim. Biophys. Acta 1166*, 171–176.
5. Hannemann, K., Puchta, V., Simon, E., Ziegler, H., Ziegler, G., and Spiteller, G. (1989) *Lipids 24*, 296–298.
6. Glass, R.L., Krick, T.P., and Eckhardt, A.E. (1974) *Lipids 9*, 1004–1008.
7. Glass, R.L., Krick, T.P., Sand, D.H., Rahn, C.H., and Schlenk, H. (1975) *Lipids 10*, 695–702.
8. Glass, R.L., Krick, T.P., Olson, D.L., and Thorson, R.L. (1977) *Lipids 12*, 828–836.
9. Scrimgeour, C.M. (1977) *J. Am. Oil Chem. Soc. 54*, 210–211.
10. Gunstone, F.D., Chakra, R., Wijesundera, R.C., and Scrimgeour, C.M. (1978) *J. Sci. Food Agric. 29*, 539–550.
11. Ota, T., and Takagi, T. (1989) *Bull. Fac. Fish. Hokkaido Univ. 40*, 193–201.
12. Glass, R.L., Krick, T.P., and Eckhardt, A.E. (1974) *Lipids 9*, 1004–1008.
13. Okajama, H., Ishii, K., and Watanabe, H. (1984) *Chem. Pharm. Bull. 32*, 3281–3286.
14. Ishii, K., Okajima, H., Koyamatsu, T., Okada, Y., and Watanabe, H. (1988) *Lipids 23*, 694–700.
15. Ishii, K., Okajima, H., Okada, Y., Konishi, H., and Watanabe, H. (1989) *Chem. Pharm. Bull. 37*, 1564–1567.
16. Ciminiello, P., Fattorusso, E., Mangoni, A., Ialenti, A., and Di Rosa, M. (1991) *Experientia 47*, 739–743.
17. Groweiss, A., and Kashman, Y. (1978) *Experientia 34*, 299.
18. Schodel, R., and Spiteller, G. (1987) *Liebigs Ann. Chem.*, 459–462.
19. Puchta, V., and Spiteller, G. (1988) *Liebigs Ann. Chem.*, 1145–1147.
20. Lie Ken Jie, M.S.F., and Syed-Rahmatullah, M.S.K. (1994) *Lipids 29*, 889–892.
21. Lie Ken Jie, M.S.F., Syed-Rahmatullah, M.S.K., and Wong, K.P. (1992) *Nat. Prod. Lett. 1*, 93–98.
22. Lie Ken Jie, M.S.F., and Ahmad, F. (1983) *J. Am. Oil. Chem. Soc. 60*, 1783–1785.
23. Miller, C., Austin, H., Posorske, L., and Gonzlez, J. (1988) *J. Am. Oil Chem. Soc. 65*, 927–931.
24. Haraldsson, G.G., Gudmundsson, B., and Almarsson, O. (1993) *Tetrahedron Lett. 34*, 5791–5794.
25. Paquot, C., and Hautfenne, A., in *IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th edn., International Union for Pure and Applied Standards, 1987, pp. 111–115, Blackwell Scientific Publications, Oxford.

[Received July 5, 1994, and in revised form October 31, 1994; Revision accepted November 15, 1994]

High-Performance Liquid Chromatography with Light-Scattering Detection and Desorption Chemical-Ionization Tandem Mass Spectrometry of Milk Fat Triacylglycerols

George A. Spanos[a,1], Steven J. Schwartz[a,*], Richard B. van Breemen[b,2] and Chien-Hua Huang[b]

[a]Southeast Dairy Foods Research Center, Department of Food Science and [b]Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695

ABSTRACT: The utility of reverse-phase high-performance liquid chromatography (HPLC), desorption chemical-ionization mass spectrometry (DCI–MS) and tandem mass spectrometry (MS/MS) for the characterization of triacylglycerols in complex mixtures has been further explored. Triacylglycerols of anhydrous bovine milk fat were separated by using two reverse-phase C_{18} HPLC columns, and eluents were monitored with an evaporative light-scattering detector. Fifty-eight fractions were resolved and analyzed by positive ion isobutane DCI–MS. The formation of protonated molecules and of major fragments corresponding to the random loss of any one of the constituent fatty acids readily identified acyl carbon numbers and the number of double bonds within each fatty acid. MS/MS was only required when the original mass spectra indicated the presence of more than one triacylglycerol or of impurities in a fraction. Protonated molecules produced by DCI were fragmented using high energy collisional activation, and the resulting ions were detected by MS/MS. Odd-chain triacylglycerols were also readily distinguished using this methodology. The positive ion DCI and MS/MS techniques described here demonstrate the usefulness of this approach for the characterization of triacylglycerols in complex mixtures.

Lipids 30, 85–90 (1995).

Milk fat contains an unusually wide range of fatty acids (1–5). Because of this complexity, the structural elucidation of milk fat triacylglycerols is a formidable task (6). Triacylglycerols can typically be analyzed by high-performance liquid chromatography (HPLC) or gas–liquid chromatography (GLC) (7). However, the low volatility of triacylglycerols requires very high GLC column temperatures (350–375°C), which can cause degradation (8), particularly with polyunsaturated triacylglycerols (9). The recent availability of evaporative light-scattering detectors (ELSD) has brought about a major advance in the detection of lipids upon HPLC separation relative to earlier types of detectors (refractive index, ultraviolet). ELSD also permits elution with solvent gradients, as is required to resolve triacylglycerol mixtures as complex as those found in milk fat (10).

A number of excellent studies have been reported which have demonstrated reverse-phase HPLC separations (11–17), as well as the application of ELSD (18–21) for triacylglycerol analysis. Only quite recently have these chromatographic methods, as well as tandem mass spectrometry (MS/MS), been used to structurally characterize mixtures of triacylglycerols (22,23). It is known that mass spectrometry (MS) of triacylglycerols using positive ion chemical ionization (CI) or desorption chemical ionization (DCI) produces protonated molecules $[M + H]^+$, as well as fragment ions corresponding to random elimination of an acyl group from the triacylglycerol, $[MH - RCOOH]^+$ (24–26). Alternatively, negative ion DCI MS/MS has been used to characterize triglyceride structures (22,23). MS/MS following collisional activation can provide fragment ions of selected protonated molecules formed during CI or DCI, thus simplifying the identification of fragments ions when more than one $[M + H]^+$ species is present.

The aim of this study was (i) to fractionate milk fat triacylglycerols by HPLC–ELSD and (ii) to characterize the triacylglycerol species present by DCI–MS and MS/MS techniques.

EXPERIMENTAL PROCEDURES

Materials. Anhydrous milk fat (butter oil) was donated by Danish Maid Butter Co. (Chicago, IL). It contained no less

[1]Present address: Applied Analytical Industries, 1206 North 23rd St., Wilmington, NC 28405.

*To whom correspondence should be addressed at the Department of Food Science, Box 7624, North Carolina State University, Raleigh, NC 27695-7624.

[2]Present address: Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 South Wood St., Chicago, IL 60612-7231.

Abbreviations: B/E, magnetic field strength/electrostatic analyzer voltage; CI, chemical ionization; DCI, desorption chemical ionization; ECN, equivalent carbon number; EI, electron impact; ELSD, evaporative light-scattering detection; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PCN, propionitrile.

Copyright © 1995 by AOCS Press

than 99.6% butter fat and no more than 0.4% moisture. 1,2-Dimyristoyl-3-lauroyl-glycerol, 1,2-dipalmitoyl-3-myristoyl-glycerol, 1,3-dioleoyl-2-stearoyl-glycerol, cholesterol, cholesterol myristate, cholesterol palmitate, cholesterol oleate and cholesterol arachidate were obtained from Sigma (St. Louis, MO). Acetonitrile, methylene chloride, acetone, hexanes and methyl-*tert*-butyl ether were purchased from Fisher (Fair Lawn, NJ). Propionitrile (PCN) was obtained from Fluka (Ronkonkoma, NY) and was purified by distillation. Ethanol was purchased from Midwest Grain Product Co. (Weston, MI). All HPLC solvents were filtered through a 0.45 μm membrane filter (Micron Separations Inc., Westboro, MA).

HPLC analysis of triacylglycerols. The HPLC setup consisted of a three-pump system, Model 510 and 501 pumps, a Gradient Controller, a U6K injector (Waters Associates, Milford, MA) and a Model ELSD-MK IIA ELSD (Varex Corp., Burtonsville, MD). Chromatograms were processed using a Dynamax HPLC Method Manager (Rainin, Woburn, MA). The analytical columns (5 μm, 25 cm × 4.6 mm i.d.) were a Spherisorb ODS-2 (Alltech Associates, Deerfield, IL) and a Zorbax C_{18} (Mac-Mod Analytical Inc., Chadds Ford, PA) connected in tandem with a Spherisorb ODS-2 precolumn (5 μm, 3.5 cm × 4.6 mm; Anspec, Ann Arbor, MI). Triacylglycerols were eluted using acetonitrile, Solvent A; PCN, Solvent B; and PCN/methylene chloride (72:28, vol/vol), Solvent C. The elution involved two linear gradient steps at a flow rate of 0.7 mL/min. The first step was from 30% Solvent A and 70% Solvent B to 5% Solvent A and 95% Solvent B in 50 min.The second step was from 5% Solvent A and 95% Solvent B to 0% Solvent A, 0% Solvent B and 100% Solvent C in 70 min. The detector drift tube temperature was 130°C, inlet pressure of nitrogen nebulizing gas was 32 psi and the detector exhaust temperature was 81–82°C. Typical milk fat separations involved 50-μL injections of milk fat solution (5 μg of milk fat per μL of PCN/methylene chloride; 3:2, vol/vol).

Isolation of triacylglycerols. Approximately 900 μg of milk fat was injected for each HPLC analysis. A flow splitter (Varex Corp.) was utilized to divide the column effluent between a fraction collector (ISCO Retriever II; ISCO, Lincoln, NE) and the detector at a split ratio of 9:1. Solvent was evaporated under a stream of N_2, fractions were reconstituted in PCN/methylene chloride (2:1, vol/vol), and aliquots were reinjected onto the HPLC to check peak purity. The remaining portion of each triacylglycerol isolate was dried (N_2 stream) and kept at −18°C until mass spectrometric analysis.

MS. Isobutane positive ion DCI mass spectra were obtained using a JEOL (Tokyo, Japan) JMS-HX110HF double-focusing mass spectrometer, equipped with a JMA-DA5000 data system and a DCI direct insertion probe. Samples were dissolved in methylene chloride (1–5 μg/μL), and 10 μL was loaded onto the tungsten DCI filament. The solvent was then evaporated and the probe inserted into the ionization source. The filament was heated at 0.25 μA/min in isobutane reagent gas so that molecules were protonated immediately upon desorption. The ion source temperature was 150°C, and the resolution was 1000 for all measurements. Based on the total ion chromatogram, 4–8 scans containing the most abundant sample ions over the region *m/z* 100–1,200 were averaged using the data system. MS/MS analyses were obtained by collisional activation of selected $[M + H]^+$ precursors, followed by linked scanning at constant magnetic field strength/electrostatic analyzer voltage (B/E), whereby the magnetic field (B) was scanned at a constant ratio to the electrostatic analyzer voltage (E). Helium was used as the collision gas in the first field-free region, and the pressure was adjusted so that the abundance of the $[M + H]^+$ precursor ion was attenuated by 70%.

RESULTS

A chromatogram showing the HPLC separation and ELSD of milk fat triacylglycerols is shown in Figure 1. The milk fat triacylglycerols were resolved into 58 peaks. Because few milk fat triacylglycerols that could be used as standards are commercially available, assignment of peaks to specific triacylglycerols is a complex task. In the present study we used positive ion DCI–MS and MS/MS with isobutane as the reagent gas to characterize the triacylglycerol(s) associated with each peak.

Although the use of isobutane as reagent gas in CI–MS has been reported to result in protonated molecules of low abundance (24,26), in our experiments DCI–MS with isobutane resulted in $[M + H]^+$ ions for all the triacylglycerols analyzed in this study. In our preliminary DCI–MS analyses of triacylglycerol standards, the relative abundances of the $[M + H]^+$ ions from 1,2-dipalmitoyl-3-myristoyl-glycerol, 1,2-dimyristoyl-3-lauroyl-glycerol and 1,3-dioleoyl-2-stearoyl-glycerol were 5, 15 and 51%, respectively.

Table 1 lists the total acyl carbon number, the number of double bonds, and the fatty acids contained in each triglyceride eluted by HPLC (peaks 1 to 58; Fig. 1). Examples of

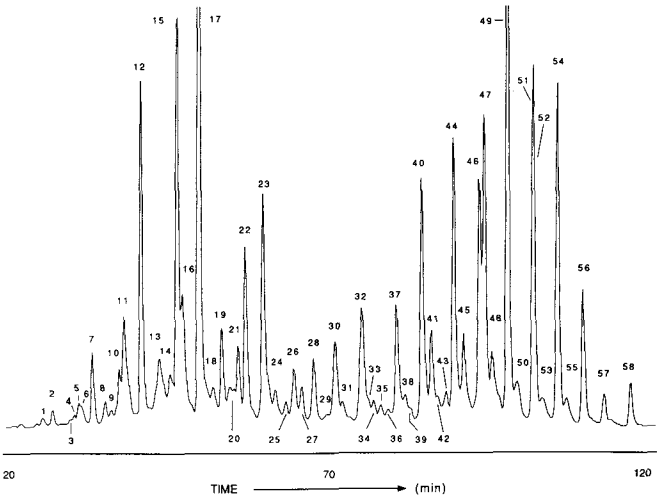


FIG. 1. High-performance liquid chromatography separation and evaporative light-scattering detection of milk fat triacylglycerols.

TABLE 1
Characterization of Milk Fat Triacylglycerols upon HPLC–ELSD by Desorption Chemical Ionization–Mass Spectrometry (MS) and MS/MS[a]

PN[b]	Acyl carbon number[c]	Triacylglycerol structures[d]
1	32:1	4,10,18:1; 6,10,16:1; 6,12,14:1; 4,12,16:1; 6,8,14:1; 8,8,16:1
2	30:0	4,10,16; 4,12,14; 6,10,14; 8,10,12; 8,8,16
	32:1	4,10,18:1; 4,16,12:1; 6,10,16:1
3	34:1	various combinations of 12, 6, 10, 8, 18:1, 16:1, 12:1
	36:2	4,12,20:2; 6,12,18:2
4	34:1	various combinations of 10, 6, 12, 14, 8, 16:1, 18:1, 14:1, 12:1, 10:1
5	34:1	4,12,18:1; 6,12,16:1; 8,10,16:1
	38:3	4,16,18:3; 6,16,16:3
6	34:1	4,12,18:1
	32:0	6,8,18
7	32:0	4,12,16; 4,14,14; 4,10,18
8	40:3	4,18:1,18:2
	38:2	6,14,18:2; 6,14:1,18:1; 14,10:1,14:1
9	36:1	6,12,18:1; 8,14,14:1; 6,14,16:1
	38:2	4,16:1,18:1
	40:3	
10	36:1	6,12,18:1; 8,10,18:1; 8,14,14:1; 6,14,16:1
	38:2	4,16,18:2; 4,16:1,18:1; 6,14,18:2
	40:3	
11	36:1	4,14,18:1; 4,16,16:1
	34:0	6,12,16; 6,12,14; 6,10,18; 4,14,16; 8,10,16
12	34:0	4,14,16; 4,12,18 (trace)
13	40:2	4,18:1,18:1; 6,16,18:2 (trace)
	35:0	
	38:1	8,12,18:1; 6,14,18:1; 10,12,16:1; 10,14,14:1
	34:0	
	36:1	
	37:1	
14	38:1	6,14,18:1; 6,16,16:1; 12,16,10:1; 4,16,18:1
	40:2	4,18:1,18:1
	36:0	
15	38:1	4,16,18:1
	36:0	4,14,18; 6,14,16; 6,12,18; 10,12,14
16	36:0	6,14,16
	38:1	4,16,18:1 (from 15)
17	36:0	4,16,16; 4,14,18 (trace)
	42:2	
	34:0	
18	40:1	8,14,18:1
	37:0	6,15,16; 4,16,17; 4,15,18; 8,14,15
	42:2	
	39:1	

[a]HPLC, high-performance liquid chromatography; ELSD, evaporative light-scattering detection.
[b]PN, peak number (numbers correspond to Fig. 1).
[c]Acylglycerol carbon number of the triacylglycerols and total number of double bonds.
[d]Underlined structures are the most abundant.

TABLE 1 (continued)

PN	Acyl carbon number	Triacylglycerol structure
19	40:1	6,16,18:1
20	40:1	6,16,18:1; 8,14,18:1; 14,16,10:1
21	40:1	4,18,18:1
	38:0	8,14,16
22	38:0	6,16,16; 6,14,18
	40:1	4,18,18:1
23	38:0	4,16,18
	44:2	8,18:1,18:1; 10,16:1,18:1; 10,16,18:2; 12,14,18:2
24	42:1	8,16,18:1; 10,14,18:1; 12,12,18:1
	39:0	
25	42:1	6,18,18:1; 8,16,18:1
	39:0	
26	40:0	10,14,16; 12,12,16; 10,12,18; 12,14,14
	42:1	6,18,18:1; 6,16,20:1 (trace)
27	40:0	8,16,16; 6,16,18; 10,14,16
	42:1	
28	40:0	6,16,18
	46:2	10,18:1,18:1
	41:0	
29	46:2	12,16,18:2; 14,14:1,18:1; 10,18:1,18:1
30	44:1	10,16,18:1; 12,14,18:1; 8,18,18:1
	40:0	4,18,18
	46:2	
31	44:1	8,18,18:1; 10,16,18:1; 12,14,18:1; 14,18,14:1; 16,16,14:1
32	42:0	10,16,16; 14,16,18
		12,14,16; 8,16,18
	50:3	14:1,18:1,18:1
33	42:0	8,16,18; 10,16,16; 10,14,18
	50:3	16,18:1,18:2
34	48:2	12,18:1,18:1; 14,16:1,18:1
	50:3	14,18:1,18:2
	42:0	10,16,16
	43:0	
35	48:2	16,14:1,18:1
	50:3	16,16:2,18:1
	42:0	6,18,18
	46:1	14,16,16:1
36	48:2	14,16,18:2; 12,18,18:2
	50:3	16,16,18:3; 14,16,20:3
	43:0	
	46:1	
37	46:1	12,16,18:1; 14,14,18:1; 10,18,18:1
	54:4	18:1; 18:1,18:2
38	46:1	12,16,18:1; 14,18,14:1; 16,16,14:1; 14,14,18:1
	54:4	10,18,18:1
	52:3	18:1,18:1,18:2
	49:2	16,16,20:3
39	52:3	16:1,18:1,18:1
	47:1	14,15,18:1
	54:4	

(continued on next page)

TABLE 1 (continued)

PN	Acyl carbon number	Triacylglycerol structures
40	44:0	12,16,16; 14,14,16; 10,16,18; 12,14,18
	52:3	16,18:1,18:2
41	50:2	14,18:1,18:1; 16,16:1,18:1
	52:3	16,18:1,18:2
	45:0	
42	50:2	14,18:1,18:1; 16,16,18:2; 16,16:1,18:1
43	48:1	14,16,18:1; 12,18,18:1
	50:2	16,16,18:2; 14,18,18:2
	52:3	
44	48:1	14,16,18:1
45	48:1	14,16,18:1
	54:3	18:1,18:1,18:1
	51:2	15,18:1,18:1
46	46:0	14,16,16; 14,14,18; 10,18,18
	54:3	18:1,18:1,18:1
	49:1	15,16,18:1; 14,17,18:1
47	52:2	16,18:1,18:1
48	52:2	16,18:1,18:1
49	50:1	16,16,18:1
50	50:1	16,16,18:1; 14,18,18:1
51	48:0	14,16,18; 12,18,18
	54:2	18,18:1,18:1 (from 52)
	51:1	16,17,18:1; 15,18,18:1
52	54:2	18,18:1,18:1
	48:0	14,16,18 (from 51)
53	54:2	18,18:1,18:1
54	52:1	16,18,18:1
55	52:1	16,18,18:1; 16,16,20:1
56	50:0	16,16,18
57	54:1	18,18,18:1; 16,20,18:1; 16,18,20:1
58	52:0	16,18,18

DCI mass spectra and tandem mass spectra are shown in Figures 2–4. The DCI–MS spectrum of HPLC peak 12 (Fig. 2) shows a protonated molecule *m/z* 611 representing a triacylglycerol structure with a total acyl carbon number of 34. The ion *m/z* 649 was formed by addition of a potassium cation to the intact molecule which facilitated the assignment of *m/z* 611 as the protonated molecule. The glass tubes in which the triacylglycerol fractions were collected and stored are the most probable source of the potassium. Fragment ions *m/z* 523, 383 and 355 were formed from the protonated molecule by loss of butyric, myristic and palmitic acid, respectively. The positions of each of the acyl groups on the glycerol backbone could not be determined from these data. Because only one triacylglycerol was detected in HPLC peak 12 and abundant fragment ions provided adequate structural information, MS/MS analyses were not obtained.

When two or more triacylglycerols or impurities, such as cholesterol or cholesteryl ester, were detected in an HPLC fraction, collisional activation of each triacylglycerol $[M + H]^+$ followed by MS/MS analysis was required to identify

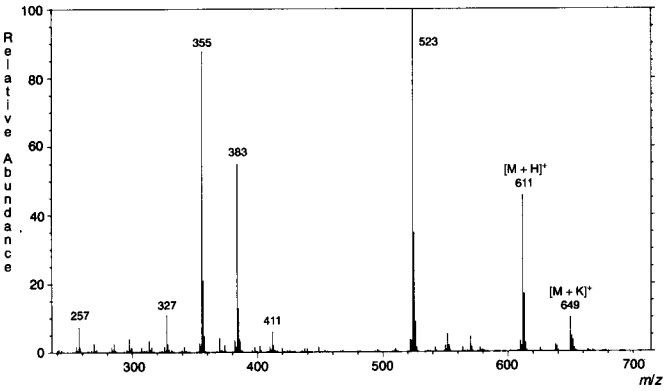


FIG. 2. Positive ion desorption chemical-ionization mass spectrum of fraction 12 (see Fig. 1) showing the presence of a single triacylglycerol (34:0). Fragment ions correspond to elimination of butyric (*m/z* 523), myristic (*m/z* 383) or palmitic (*m/z* 355) acid from the protonated molecule.

structurally significant fragment ions specific to that compound. For example, the DCI mass spectrum of peak 16 (Fig. 3) showed two protonated molecules *m/z* 665 and 639, of approximately equal abundance. MS/MS analysis of the $[M + H]^+$ ion of *m/z* 665 (Fig. 4a) showed $[MH - RCOOH]^+$ fragment ions at *m/z* 577, 409 and 383, which identified this triacylglycerol as 4,16,18:1. MS/MS analysis of the $[M + H]^+$ ion of *m/z* 639 (Fig. 4b) showed fragment ions corresponding to loss of C_6, C_{14} or C_{16} fatty acid. Therefore, the protonated ion *m/z* 639 was identified as being due to a 6,14,16 triacylglycerol.

Triacylglycerols with branched-chain, odd-acyl carbon numbers were readily identified and characterized by MS/MS. For example, MS/MS of the $[M + H]^+$ ion *m/z* 653 (37:0 triacylglycerol; peak 18) gave ions *m/z* 565, 537, 397 and 369, which were formed by elimination of 4, 6, 16 or 18 carbon fatty acids from a mixture of isomeric triacylglycerols. The fragment ions *m/z* 411 and 383 represented branched, odd-chain fatty acids with 15 and 17 carbons, respectively.

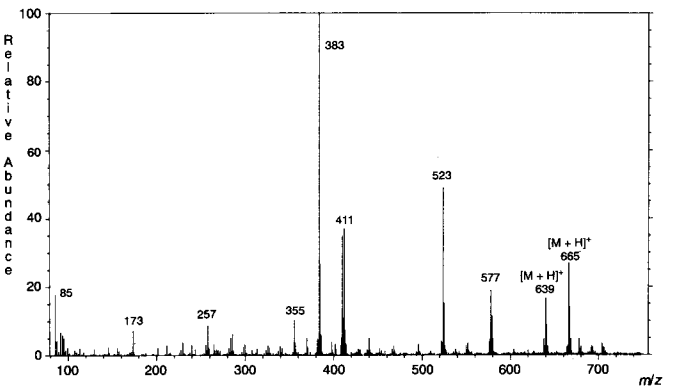


FIG. 3. Positive ion desorption chemical-ionization mass spectrum of fraction 16 (see Fig. 1) showing the protonated ions of two triacylglycerols (38:1 and 36:0).

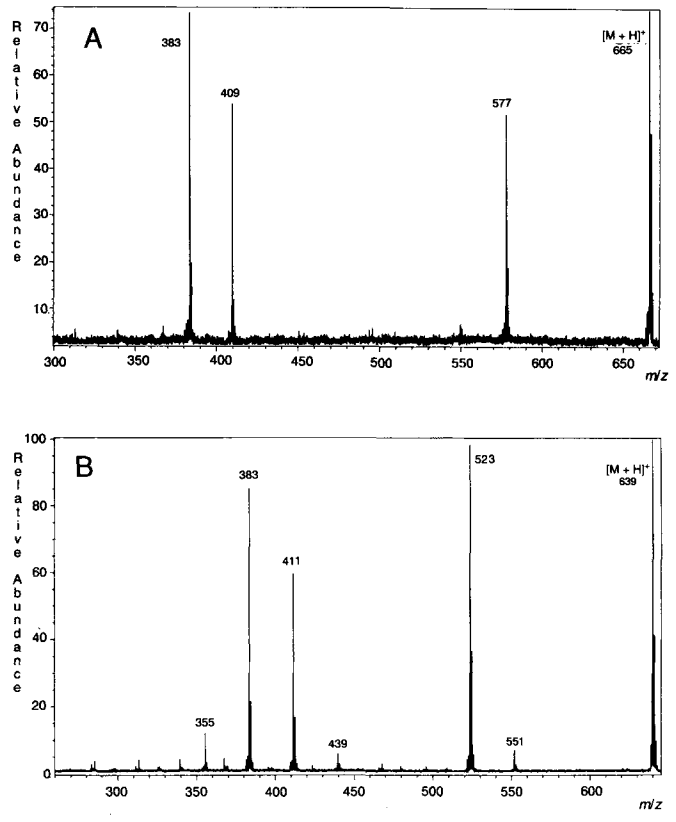


FIG. 4. Tandem mass spectrum of the $[M + H]^+$ precursor ion of *m/z* 665 (Panel A) and of *m/z* 639 (Panel B) shown in Figure 3. Fragment ions of the 38:1 triacylglycerol (Panel A) correspond to a fatty acid composition of 4,16,18:1. Fragment ions of the 36:0 triacylglycerol (Panel B) correspond to elimination of C_6 (*m/z* 523), C_{14} (*m/z* 411) and C_{16} (*m/z* 383) fatty acids from $[M + H]^+$.

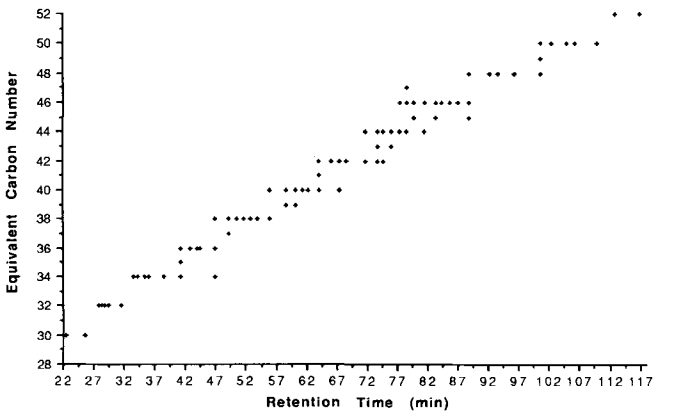


FIG. 5. Plot of retention times vs. equivalent carbon numbers (ECN) of milk fat triacylglycerols.

Based on the relative abundances of these fragment ions, most of the triacylglycerols in this mixture were 6,15,16 and 4,16,17, while 4,15,18 and 8,14,15 were present only as minor components.

Figure 5 shows the relationship between HPLC retention times and equivalent carbon numbers (ECN) of milk fat triacylglycerols. Triacylglycerols that differed by more than one ECN were typically resolved. Triacylglycerols of equal ECN eluted over a 5–10 min range.

DISCUSSION

Although HPLC separations of milk fat triacylglycerols have previously been carried out successfully (14,17), GLC methods have typically been used to characterize the fatty acid profiles of triglycerides upon enzymatic hydrolysis rather than analyze the triglyceride molecules themselves. Only quite recently have milk fat triglyceride structures been directly elucidated using MS/MS techniques (22). In earlier studies based on electron impact (EI) ionization of triacylglycerols, molecular ions of sufficient intensity to provide molecular weight information were usually not obtained (24,26). However, $[M + H]^+$ ions of high abundance can be obtained for both low- and high-molecular weight triacylglycerols by DCI–MS. The DCI fragmentation patterns of triacylglycerols are different from those seen upon EI ionization. For example, typical EI–MS fragment ions such as $[RCO]^+$ and $[RCO + 74]^+$ (27) are not observed in the DCI mass spectra. Ions corresponding to protonated fatty acids are sometimes detected. The most intense $[RCOOH_2]^+$ ions were observed at *m/z* 257, 283 and 285 (corresponding to palmitic, oleic and stearic acid, respectively), whereas protonated butyric acid was rarely seen. Because $[RCOOH_2]^+$ ions were not observed in tandem mass spectra of protonated triacylglycerols, these ions are probably formed by loss of neutral fatty acids from triacylglycerols in the ion source, followed by protonation by the reagent gas.

In the DCI mass spectra of HPLC peaks 25 through 58 (see Fig. 1), abundant ions were detected at *m/z* 369 and 391. Peaks 44 through 49 contained ions *m/z* 387 which correspond to protonated cholesterol. The DCI mass spectra of cholesteryl ester standards, including cholesteryl myristate, cholesteryl palmitate, cholesteryl oleate and cholesteryl arachidate, showed protonated molecules as base peaks at *m/z* 369, but no other abundant ions. Chromatography of these cholesteryl esters showed that these compounds eluted within the range of retention times between peaks 25 through 58.

In other CI or DCI mass spectrometric investigations, ammonia has been most widely used as reagent gas in triacylglycerol analysis. In positive ion CI and DCI mass spectra, abundant $[M + H]^+$ and $[M + NH_4]^+$ ions were detected (24,26), but the ion signal intensity was divided between $[M + H]^+$ and $[M + NH_4]^+$ species, thereby reducing sensitivity. Therefore, isobutane is preferred over ammonia as a positive ion DCI reagent gas for triacylglycerol analysis.

In negative ion DCI, ammonia has been used to facilitate the formation of deprotonated triacylglycerols with little or no fragmentation, thereby resulting in high sensitivity (22,23). However, in the absence of fragmentation in negative ion DCI, a second analysis by collisional activation in MS/MS is required to obtain diagnostically significant fragment ions. When using positive ion DCI, as used in this in-

vestigation, MS/MS measurements are only necessary if the sample contains several triacylglycerols or impurities.

The location of fatty acid double bonds in the carboxylate ions can be determined by using high energy collisional activation to induce charge-remote fragmentation followed by MS/MS analysis, such as linked scanning at constant B/E (28,29). However, because of the great number of HPLC fractions we obtained and due to limitations in mass spectrometer time, a systematic characterization of the acyl groups to locate the sites of unsaturation was not carried out.

Within each cluster of HPLC peaks with the same ECN (Fig. 5), triacylglycerols containing polyunsaturated fatty acids eluted earlier than less unsaturated or saturated triacylglycerols. Nevertheless, the ECN model cannot provide a comprehensive description of the milk fat triacylglycerol profile, when a gradient chromatographic elution system of high selectivity is employed. Other models (i.e., theoretical carbon number), attempting to relate the elution order with the triacylglycerol structure, were not evaluated in this study.

After careful examination of peak purity, possible carry over, and of the DCI–MS and MS/MS spectra, our data suggest that the 4,10,18:1 triacylglycerol of peak 1 is different from the 4,10,18:1 structure of peak 2. Other examples include the 14,16,18:1 triacylglycerol of peaks 44 and 45 and the 16,18,18:1 structure of peaks 54 and 55. Because all these triacylglycerols contain unsaturated fatty acids, different chromatographic retention times for isomer pairs might represent differences in the location or geometry of double bonds rather than in the positional distribution of fatty acids on the glycerol backbone. This is most likely the case for the 18:1,18:1,18:1 triacylglycerols appearing in both the 45 and 46 HPLC peaks. Other examples of HPLC separations of positional isomers of triacylglycerols have been reported (25,26).

Recently, the composition of triacylglycerols of butterfat fractions obtained by an industrial melt crystallization process were characterized (30). Triacylglycerol structures of the fractions were determined by using a combination of chromatographic techniques including both HPLC and gas chromatographic analyses. Profiles of milk fat that were presented (30) were similar and complementary to those reported in the present investigation. The advantages of the methodology we report here include the structural characterization of purified triacylglycerols directly by DCI–MS and MS/MS techniques without prior modification or derivatization.

ACKNOWLEDGMENTS

The Southeast Dairy Foods Research Center at North Carolina State University (Raleigh, NC) provided financial support for this work. This is paper No. FSR93-42 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh.

REFERENCES

1. Christie, W.W. (1987) in *Recent Advances in Chemistry and Technology of Fats and Oils* (Hamilton, R.J. and Bhati, A., eds.) pp. 57–78, Elsevier Science Publishing Co., New York.
2. Christie, W.W. (1981) in *Lipid Metabolism in Ruminant Animals* (Christie, W.W., ed.) pp. 93–191, Pergamon Press, New York.
3. Myher, J.J. and Kuksis, A. (1982) *Can. J. Biochem. 60*, 638–650.
4. Christie, W.W. (1983) *Developments in Dairy Chemistry 2. Lipids*, pp. 1–35, Applied Science Publishers, New York.
5. Patton, S., and Jenson, R.G. (1975) *Prog. Chem. Fats Other Lipids 14*, 162–277.
6. Bhati, A. (1987) in *Recent Advances in Chemistry and Technology of Fats and Oils* (Hamilton, R.J., and Bhati, A., eds.) pp. 13–30, Elsevier Science Publishing Co., New York.
7. Geeraert, E., and Sandra, P. (1987) *J. Am. Oil Chem. Soc. 64*, 100–105.
8. Murata, T., and Takahashi, S. (1977) *Anal. Chem. 49*, 728–731.
9. Rezanka, T., and Mares, P. (1991) *J. Chromatogr. 542*, 145–159.
10. Robinson, J.L., and Macrae, R. (1984) *J. Chromatogr. 303*, 386–390.
11. Deffense, E. (1984) *Rev Franc. Corps Gras 31*, 123–129.
12. Dotson, K.D., Jerrell, J.P., Picciano, M.F., and Perkins, E.G. (1992) *Lipids* 27, 933–939.
13. Schulte, V.E. (1981) *Fette Seifen Anstrichm. 83*, 289–291.
14. Frede, E., and Thilele, H. (1987) *J. Am. Oil Chem. Soc. 64*, 521–528.
15. Nikolova-Damyanova, B., Christie, W.W., and Herslöf, B. (1990) *J. Am. Oil Chem. Soc. 67*, 503–507.
16. Phillips, F.C., Erdahl, W.L., Nadenicek, J.D., Nutter, L.J., and Schmit, J.A. (1984) *Lipids 19*, 142–150.
17. Maniongui, C., Gresti, J., Bugaut, M., Gauthier, S., and Bezard, J. (1991) *J. Chromatogr. 543*, 81–103.
18. Stolyhowo, A., Colin, H., Martin, M., and Guiochon, G. (1984) *J. Chromatogr. 288*, 253–275.
19. Stolyhwo, A., Colin, H., and Guiochon, G. (1985) *Anal. Chem.. 57*, 1342–1354.
20. Robinson, J.L., Tsimidou, M., and Macrae, R. (1985) *J. Chromatogr. 324*, 35–51.
21. Herslöf, B., and Kindmark, G. (1985) *Lipids 20*, 783–790.
22. Currie, G.J., and Kallio, H. (1993) *Lipids 28*, 217–222.
23. Kallio, H., and Currie, G.J. (1993) *Lipids 28*, 207–215.
24. Murata, T. (1977) *Anal. Chem. 49*, 2209–2213.
25. Kuksis, A., Myher, J.J. and Marai, L. (1985) *J. Am. Oil Chem Soc. 62*, 762–767.
26. Kuksis, A. Myher, J.J., and Marai, L. (1984) *J. Am. Oil Chem. Soc. 61*, 1582–1589.
27. Weintraub, S.T. (1990) in *Mass Spectrometry of Biological Materials* (McEwen, C.N., and Larsen, B.S., eds.) pp. 257–286, Marcel Dekker, New York.
28. van Breemen, R.B. (1992) in *Mass Spectrometry in the Biological Sciences: A Tutorial* (Gross, M.L., ed.) pp. 443–451, Kluwer Academic Publishers, Dordrecht.
29. Adams, J. (1990) *Mass Spectrom. Rev. 9*, 141–186.
30. Laakso, P.H., Nurmela, K.V.V., and Homer, D.R. (1992) *J. Agr. Food Chem. 40*, 2472–2482.

[Received September 9, 1993, and in revised form October 31, 1994; Revision accepted November 5, 1994]

Comparative Studies of Metabolism of 4-Desmethyl, 4-Monomethyl and 4,4-Dimethyl Sterols in *Manduca sexta*

James A. Svoboda[a,*], Samir A. Ross[b] and W. David Nes[c]

[a]Insect Neurobiology & Hormone Laboratory, ARS, USDA, Beltsville, Maryland 20705, [b]Department of Pharmacy, University of Mississippi, Oxford, Mississippi 38677 and [c]Department of Chemistry & Biochemistry, Texas Tech University, Lubbock, Texas 79409

ABSTRACT: To investigate the metabolism and possible deleterious effects of 4-methyl and 4,4-dimethyl steroids in *Manduca sexta*, the 4,4-dimethyl sterols lanosterol and cycloartenol, the 4-methyl sterol obtusifoliol and the 4,4-dimethyl pentacyclic triterpenoid α-amyrin were fed in an artificial agar-based diet at various concentrations. Utilization and metabolism of these four compounds were compared with sitosterol, stigmasterol, brassicasterol, ergosterol and 24-methylenecholesterol, 24-alkyl sterols that are readily dealkylated and converted to cholesterol in *Manduca* and in most phytophagous insects. None of the 4-methylated compounds significantly inhibited development except at very high dietary concentrations. The Δ^{24}-bonds of lanosterol and cycloartenol were effectively reduced by the *Manduca* Δ^{24}-sterol reductase enzyme, as is the Δ^{24}-bond of desmosterol which, in most phytophagous insects, is an intermediate in the conversion of sitosterol, stigmasterol and other C_{28} and C_{29} phytosterols to cholesterol. On the other hand, the 24-methylene substituent of obtusifoliol was not dealkylated. Each of the 4-desmethyl C_{28} and C_{29} sterols was readily converted to cholesterol, and a significant amount of 7-dehydrocholesterol was derived from ergosterol metabolism. The reason for the differences in substrate specificity of these sterols is not clear, but the information may be useful in the development of new, specific, mechanism-based inhibitors of sterol metabolism.

Lipids 30, 91–94 (1995).

A considerable body of literature on the utilization and metabolism of 4-desmethyl sterols in insects exists, and several reviews provide thorough discussion of this area (1–3). This knowledge is based primarily on studies on metabolism of C_{28} and C_{29} phytosterols (e.g., campesterol, sitosterol and stigmasterol), which are the most prevalent sterols in plant material and comprise the majority of sterols ingested by phytophagous insects (4). Insects are unable to biosynthesize sterols, and most species utilize cholesterol as a biosynthetic precursor for the steroid molting hormones, the ecdysteroids (5). As little or no cholesterol is present in most plant material, the majority of phytophagous insects rely on their ability to dealkylate the one- or two-carbon alkyl groups attached to C-24 on the sterol side chain of phytosterols to produce the necessary cholesterol (1).

The sterols of wheat seedlings treated with the systemic fungicide fenpropimorph consisted mainly of 9ß,19-cyclopropyl sterols. Feeding *Locusta migratoria* with treated wheat seedlings having this greatly altered sterol composition produced deleterious effects on larval development and reduced the ecdysteroid content of eggs (6,7). Also, feeding impure lanosterol in a well-defined diet to *Heliothis zea* greatly limited larval development (8). Genetically manipulating sterol biosynthesis in selected crop plants could possibly limit the availability of utilizable sterols for phytophagous insects and thus provide a new approach for insect control. Because 4,4-dimethyl and 4-methyl sterols are involved in phytosterol biosynthesis, it was of interest to determine whether certain 4-methylated sterols had adverse effects when fed at high concentrations to a phytophagous insect, as well as the extent to which the insect metabolized them. To this end, several highly purified 4-methylated compounds, including cycloartenol, lanosterol and obtusifoliol and the pentacyclic α-amyrin, were each fed as the sole added sterol in an artificial diet to the tobacco hornworm, *Manduca sexta*. Their metabolism and effects on insect development were compared with the well-studied 4-desmethyl sterols sitosterol, stigmasterol, brassicasterol and 24-methylenecholesterol, each of which is readily converted to cholesterol in *Manduca* (9,10). Cholesterol was also included as a control supplement, and the metabolism of ergosterol, previously not studied as a dietary sterol in *Manduca*, was examined.

MATERIALS AND METHODS

Manduca sexta larvae were reared on an artificial diet prepared in 100 mL portions as previously described (11), ex-

*To whom correspondence should be addressed at the Insect Neurobiology & Hormone Laboratory, USDA, ARS, PSI, Bldg. 467, BARC-East, Beltsville, MD 20705-2350.

Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

cept that brewer's yeast was not included and the casein was extracted six times rather than three in order to minimize endogenous sterol content as much as possible. Thus, wheat germ, which is necessary at a minimum concentration of 3 g per 100 mL of diet to obtain satisfactory larval growth, is the only significant source of sterol in the artificial diet. Three grams of wheat germ contain *ca.* 10 mg of sterol, the equivalent of 100 ppm of endogenous sterols composed mainly of sitosterol (~60%) and campesterol (~25%), about 1% cholesterol and several other minor components. Test compounds were coated on the dry diet ingredients in dichloromethane to provide the desired concentration in the complete diet. For metabolic studies and sterol analysis, newly hatched larvae were fed each of the compounds at a concentration of 260 ppm (26 mg/100 mL) of added compound. Six larvae were included in each test. After the gut was purged, prepupae were weighed and stored in methanol at –20°C until analyzed. To the extent that the supply of the compounds allowed, each 4-methylated sterol was included in the diet at increasing concentrations of 520, 1040 and 2080 ppm in order to observe possible effects on larval growth and development. All test compounds except sitosterol (synthesized in our laboratory) were isolated from natural sources, and the purity of each was as follows: cholesterol (99%), sitosterol (99%), stigmasterol (98%), brassicasterol (100%), ergosterol (100%), 24-methylenecholesterol (97%), cycloartenol (97%), lanosterol (100%), obtusifoliol (95%) and α-amyrin (100%). [2-^{3}H]Lanosterol (2.17×10^5 dpm/mg sp. act.) was also fed in one experiment to provide additional verification of any metabolism that might occur. [2-^{3}H]Lanosterol was prepared according to the method of Le and Nes (12).

Prior to sterol analysis, insects were minced and homogenized in CH_3OH/H_2O/5% KOH (80:10:10, by vol) and refluxed for three hours. An equal volume of H_2O was added and the nonsaponifiable material was extracted four times with ether; the ether fractions were combined and the solvent was removed on a rotoevaporator. 4-Desmethyl, 4-monomethyl and 4,4-dimethyl sterols were purified and separated by preparative thin-layer chromatography (TLC) on Analtech (Newark, DE) silica gel plates (250 μm) developed in benzene/ether (85:15, v/v). The respective bands were scraped from the plate and eluted with CH_3OH. The fractions were analyzed isothermally at 245°C by gas–liquid chromatography (GLC) on a packed 3% SE-30 column on an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA). High-performance liquid chromatograph (HPLC) analysis of these fractions was performed on a 25 cm C_{18}-Whatman (Clifton, NJ) reversed-phase column (0.5 μm) at 40°C with a solvent system of CH_3OH/H_2O (96:4, v/v) flowing at 1.5 mL/min. Gas–liquid chromatography/mass spectrometric (GC/MS) analysis of each free sterol peak was performed on a table-top Hewlett-Packard model 5870 mass detector equipped with a DB-1-coated 15 m × 0.32 mm capillary column (0.25 μm film). Electron impact spectra were collected at 70 eV.

RESULTS

The analyses of sterols and triterpenoid from *Manduca* prepupae isolated from larvae fed the various sterols added at 260 ppm in the artificial diet are summarized in Table 1. Normal growth occurred with each compound at this concentration. In the cholesterol-fed controls, 87% of the sterols was cholesterol. As expected, cholesterol was the major component of the sterols from insects fed diets supplemented with the 4-desmethyl 24-alkyl sterols sitosterol, stigmasterol, brassicasterol, ergosterol and 24-methylenecholesterol. 7-Dehydrocholesterol (cholesta-5,7-dienol) comprised 18.2% of the sterols from ergosterol-fed insects. In the lanosterol-fed insects, lanosterol (31.3%) and dihydrolanosterol (40.4%) were the major sterols. The presence of 18.7% cholesterol in the sterols from these insects indicates an efficient uptake and dealkylation of the endogenous 4-desmethyl dietary sterols, such as sitosterol and campesterol. Verification of the conversion of [^{3}H]lanosterol to [^{3}H]dihydrolanosterol provided further evidence for the reduction of the Δ^{24}-bond of lanosterol by *Manduca* (45% conversion). No other product of [^{3}H]-lano-sterol metabolism was detected by TLC or HPLC, indicating only the Δ^{24}-bond was transformed by the insect. Similarly, in the cycloartenol-fed insects, the Δ^{24}-bond was efficiently reduced to provide cycloartanol as a major sterol (16.0%), with cycloartenol comprising 51.0% of the total sterols. The 24-methylenecycloartenol identified (1.7%) was from the starting material. Again, cholesterol was the major (20.1%) 4-des-methyl sterol, as was the case with lanosterol-fed insects.

Obtusifoliol was the major sterol (46.6%) isolated from insects fed that sterol, and cholesterol (35.8%) was the most abundant 4-desmethyl sterol. The Δ^7-obtusifoliol came from the starting material. There was no indication of metabolism of obtusifoliol by *Manduca*. α-Amyrin constituted only 33.5% of the total "sterols" in the insects fed this triterpenoid, suggesting that it was not absorbed as well as the endogenous 4-desmethylsterols. Cholesterol (35.2%) was the major sterol in these insects.

The differences in levels of 4-desmethyl sterols in the 4-methyl- and 4,4-dimethyl sterol-fed insects are worth noting. In the lanosterol- and cycloartenol-fed insects, the total desmethyl sterols were 26.2 and 29.6% of the total sterols, respectively. In contrast, desmethyl sterols comprised 44.6% of the total sterols in obtusifoliol-fed insects and 66.5% of the total in α-amyrin-fed *Manduca*.

Insufficient obtusifoliol was available to examine the possible effects on development at concentrations higher than 260 ppm. Cycloartenol was fed at dietary concentrations of 520 and 1040 ppm, and, although some slowing of larval growth occurred at 1040 ppm, normal prepupae and pupae resulted. Sufficient material was available to test α-amyrin up to 2080 ppm. Larval growth was delayed at 1040 ppm, but four normal pupae were obtained from the six larvae, and three normal adults emerged. When fed 2080 ppm α-amyrin, larval development was greatly inhibited, two prepupae were

TABLE 1
Relative Percentages of Major Sterols and Triterpenoid of *Manduca* Fed Various Sterols or Triterpenoid[a]

Dietary sterol or triterpenoid		Percent of total sterol and triterpene		
		4-Desmethyl	4-Monomethyl	4,4-Dimethyl
Cholesterol[b]	Cholesterol	87.2	—	—
	Desmosterol	4.4	—	—
	Campesterol	4.3	—	—
	Sitosterol	4.1	—	—
Sitosterol	Cholesterol	77.1	—	—
	Desmosterol	Trace	—	—
	Campesterol	5.5	—	—
	Sitosterol	17.4	—	—
Stigmasterol	Cholesterol	70.1	—	—
	Campesterol	7.3	—	—
	Stigmasterol	11.2	—	—
	Sitosterol	8.3	—	—
	Unknown	3.1	—	—
Ergosterol	Cholesterol	49.1	—	—
	Cholesta-5,7-dienol	18.2	—	—
	Ergosterol	7.1	—	—
	Campesterol	10.1	—	—
	Sitosterol	10.3	—	—
	Stigmasterol	3.2	—	—
Brassicasterol	Cholesterol	64.2	—	—
	Brassicasterol	14.4	—	—
	Campesterol	10.3	—	—
	Sitosterol	10.1	—	—
24-Methylenecholesterol	Cholesterol	73.6	—	—
	Desmosterol	4.3	—	—
	24-Methylenecholesterol	11.6	—	—
	Campesterol	7.0	—	—
	Sitosterol	3.5	—	—
Lanosterol	Cholesterol	18.7	—	Lanosterol 31.3
	Desmosterol	1.5	—	Dihydrolanosterol 40.4
	Campesterol	3.1	—	—
	Sitosterol	2.9	—	—
Cycloartenol	Cholesterol	20.1	—	Cycloartanol 16.0
	Desmosterol	1.5	—	Cycloartenol 51.0
	Campesterol	4.2	—	24-Methylenecycloartenol 1.7
	Sitosterol	3.8	—	—
Obtusifoliol	Cholesterol	35.8	Obtusifoliol 46.6	—
	Desmosterol	2.8	Δ^7-Obtusifoliol 6.0	—
	Campesterol	3.7	—	—
	Sitosterol	2.3	—	—
α-Amyrin	Cholesterol	35.2	—	α-Amyrin 33.5
	Desmosterol	5.5	—	—
	Campesterol	10.6	—	—
	Sitosterol	15.2	—	—

[a]Analysis of each compound made by gas–liquid chromatography and verified by gas chromatography/mass spectrometry.
[b]Trivial and systematic names: Cholesterol, cholest-5-en-3ß-ol; desmosterol, cholesta-5,24-dien-3ß-ol; campesterol (24*R*)-24-methylcholest-5-en-3ß-ol; sitosterol, (24*R*)-24-ethylcholest-5-en-3ß-ol; stigmasterol, (24*S*)-24-ethylcholesta-5,22-*E*-dien-3ß-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3ß-ol; ergosterol, (24*R*)-24-methylcholesta-5,7,22-*E*-trien-3ß-ol; brassicasterol, (24*R*)-24-methylcholesta-5,22-*E*-dien-3ß-ol; 24-methylenecholesterol, 24(*E*) methylenecholest-5-en-3ß-ol; lanosterol, 4,4,14- trimethyl-5α-cholesta-8,24-dien-3ß-ol; dihydrolanosterol, 4,4,14-trimethyl-5α-cholest-8-en-3ß-ol; cycloartenol, 4,4,14-trimethyl-9ß,19-cyclo-5α-cholest-24-en-3ß-ol; cycloartanol, 4,4,14-trimethyl-9ß,19-cyclo-5α-cholestan-3ß-ol; obtusifoliol, 4α,14α-dimethyl-24-methylene-5α-cholest-8-en-3ß-ol (Ref. 13).

obtained, but no adults emerged. With adequate material available, lanosterol was the most extensively tested of the 4-methylated compounds. In three tests at 1040 ppm, some delay of larval development was noted, but pupae and some adults were obtained. At 2080 ppm, larval development was greatly retarded in each of six tests, but in only one test were no prepupae obtained. However, no adult emergence occurred in any of the six experiments with lanosterol at 2080 ppm.

DISCUSSION

As expected, cholesterol was the major sterol in *Manduca* prepupae from larvae fed 4-desmethyl sterols. In ergosterol-fed insects, occurrence of 7-dehydrocholesterol as a major metabolite (18.2%) reflected the additional nuclear double bond. 7-Dehydrocholesterol has been identified as a major sterol in the flour beetle (*Tribolium confusum*), regardless of dietary sterol tested (14). 7-Dehydrocholesterol is a precursor

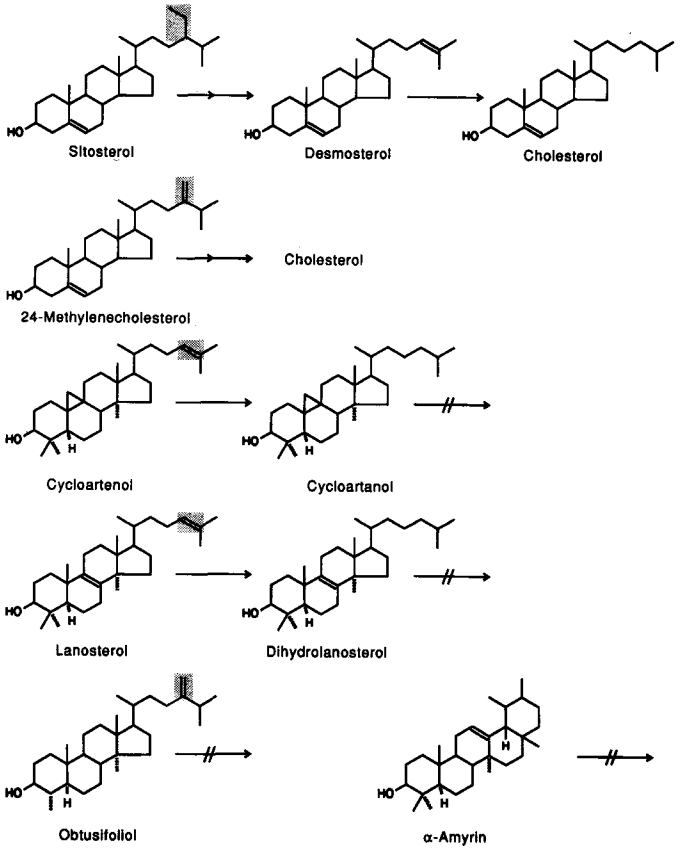


FIG. 1. Metabolism of representative 4-desmethyl, 4-methyl and 4,4-dimethyl sterols in *Manduca sexta*.

for the molting hormones (ecdysteroids), and outside of the prothoracic glands, is usually only present in *Manduca* in trace amounts (15).

The analyses of sterols from *Manduca* prepupae reared on diet supplemented with 260 ppm of the 4-methylated sterols revealed that metabolism of 4-methylated sterols does occur, but is limited to reduction of the Δ^{24}-bonds of cycloartenol and lanosterol. Even with the 4,4,14-trimethyl structures, the Δ^{24}-sterol reductase enzyme is quite effective in reducing these side-chain double bonds. No further metabolism of these two sterols was apparent. On the other hand, although 24-methylenecholesterol is readily dealkylated and converted to cholesterol in this insect, the 24-methylene of obtusifoliol was not dealkylated. It is interesting to note that the Δ^{24}-reductase enzyme is able to reduce the Δ^{24}-bond of 4,4-dimethyl sterols, but the enzymes that dealkylate 24-methylene groups are unable to dealkylate this group from a 4-monomethyl sterol. In addition, the pentacyclic triterpenoid α-amyrin was incorporated unchanged by *Manduca*. The comparative metabolism of the steroids used in this study is illustrated in Figure 1.

The differences in total desmethyl sterols found in *Manduca* fed the various 4-methyl steroids indicate that lanosterol and cycloartenol are most readily absorbed and incorporated in the tissues. The insect more readily takes up the endogenous 4-desmethyl sterols (sitosterol, campesterol) than obtusifoliol or α-amyrin. The reason for these differences is not clear, but certainly it appears that a pentacyclic triterpenoid is least preferred, and that the sterols with a side chain most similar to the cholesterol side chain are preferentially absorbed.

It has been well documented that certain insects are capable of selective sterol uptake from the diet (16). Likewise, egg production can be affected when adult locusts are fed wheat seedlings in which the sterol content has been greatly altered from the usual C_{28} and C_{29} phytosterols to mostly 9ß,19-cyclopropylsterols (6,7). In the present study, lanosterol, which differs from cycloartenol only by lacking the 9ß,19-cyclopropyl group, was inhibitory at the highest concentration tested. Perhaps, if the accumulation of the normal plant sterol intermediate cycloartenol, or another related compound, could be induced in the plant by altering specific enzyme activity *via* genetic engineering or by other means such as a steroid metabolism inhibitor, development of larvae of certain phytophagous plant pests could be disrupted.

ACKNOWLEDGMENTS

We thank Lynda J. Lyska and Owen J. Duncan III for technical assistance. W.D.N. thanks the Texas Tech Biotechnology Minigrant Program and the Welch Foundation for partial support of this research.

REFERENCES

1. Svoboda, J.A., and Thompson, M.J. (1985) in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* (Kerkut, G.A., and Gilbert, L.I., eds.) Vol. 10, pp. 137–175, Pergamon, New York.
2. Rees, H.H. (1989) in *Ecdysone* (Koolman, J., ed.) pp. 152–160, Georg Thieme Verlag, New York.
3. Svoboda, J.A., Weirich, G.F., and Feldlaufer, M.F. (1991) in *Physiology and Biochemistry of Sterols* (Patterson, G.W., and Nes, W.D., eds.) pp. 294–323, American Oil Chemists' Society, Champaign.
4. Nes, W.R., and McKean, M.L. (1977) *Biochemistry of Steroids and Other Isopentenoids*, pp. 325–410, University Park Press, Baltimore.
5. Grieneisen, M.L. (1994) *Insect Biochem. Molec. Biol. 24*, 115–132.
6. Costet, M.-F., El Achouri, M., Charlet, M., Lanot, R., Benveniste, P., and Hoffmann, J.A. (1987) *Proc. Natl. Acad. Sci. USA 84*, 643–647.
7. Charlet, M., Roussel, J.-P., Rinternecht, E., Berchtold, J.-P., and Costet, M.-F. (1988) *J. Insect Physiol. 34*, 787–796.
8. Ritter, K.S. (1986) *Arch. Insect Biochem. Physiol. 3*, 349–362.
9. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1967) *Life Sci. 6*, 395–404.
10. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1972) *Lipids 7*, 156–158.
11. Kaplanis, J.N., Thompson, M.J., Yamamoto, R.T., Robbins, W.E., and Louloudes, S.J. (1966) *Steroids 8*, 605–623.
12. Le, P.H., and Nes, W.D. (1986) *Chem. Phys. Lipids 40*, 57–69.
13. Parker, S.R., and Nes, W.D. (1992) in *Regulation of Isopentenoid Metabolism* (Nes, W.D., Parish, E.J., and Trzaskos, J.M., eds.) pp. 110–145, American Chemical Society, Washington, D.C.
14. Svoboda, J.A., Robbins, W.E., Cohen, C.F., and Shortino, T.J. (1972) in *Insect and Mite Nutrition* (Rodriguez, J.G., ed.) pp. 505–516, North Holland, Amsterdam.
15. Robbins, W.E., Kaplanis, J.N., Svoboda, J.A., and Thompson, M.J. (1971) *Annu. Rev. Entomol. 16*, 53–72.
16. Thompson, M.J., Louloudes, S.J., Robbins, W.E., Waters, J.A., Steele, J.A., and Mosettig, E. (1963) *J. Insect Physiol. 9*, 615–622.

[Received August 31, 1994, and in revised form October 28, 1994; Revision accepted December 2, 1994]

Hypothesis: An Amino Acid Sequence in Lipoprotein Lipase Codes for Its Degradation by Ca^{2+}-Dependent Proteases

Dear Sir:

Lipoprotein lipase (E.C. 3.1.1.34; LPL) is a glycosylated homodimer bound to the intraluminal surface of capillaries in most extrahepatic tissues and is responsible for the hydrolysis of circulating triacylglycerols into fatty acids and monoacylglycerol. Its activity is particularly high in adipose tissue and muscle, corresponding to their use of triacylglycerol-derived fatty acids for storage and/or oxidation. The regulation of LPL is tissue-specific. The enzyme activity is high in adipose tissue in the postprandial state and low during fasting, whereas the converse occurs in muscle (1). The degradation of LPL could be a key regulatory mechanism of the level of tissue LPL activity. Four pathways of LPL degradation have been suggested: (i) newly synthetized LPL is degraded in the lysosome before its secretion; (ii) the secreted enzyme can be re-internalized by parenchymal cells to enter the lysosomes and be degraded; (iii) the endothelium-bound enzyme can be detached and can reach the liver through the circulation; and (iv) the degradation of LPL can occur in the endoplasmic reticulum (2). Little is known, however, about the possible molecular mechanisms involved in these processes.

It has been found that LPL contains a sequence rich in the amino acids proline (P), glutamate (E), serine (S) and threonine (T), which is known to modulate protein degradation (3). The PEST-rich sequence of LPL spans 23 to 52 amino acids depending on the species and contains as much as 50% of PEST residues, which is about twice their frequency in the whole protein. In addition, the basic residues arginine, lysine and histidine are absent in the PEST-rich region but more frequent in the flanking regions than in the whole LPL sequence (4,5) (Fig. 1). Thus LPL meets the criterion of a protein characterized as having a PEST sequence. A PEST-rich sequence is a typical feature of rapidly degraded proteins (3), so it is not surprising that LPL contains one, since this enzyme has a half-life in various cell lines of about one hour (2). A PEST sequence is present in some proteins that are not rapidly degraded, but it has been suggested that, in these few cases, it is absent from the surface of the protein when it assumes its normal tertiary structure (3). The tertiary structure of LPL is not known, but it is frequently compared with human pancreatic lipase which is very homologous to LPL and has been crystallized (4). Based on this crystal structure, a model of the homodimeric LPL has recently been published in which one side of the carboxy-terminal region of one monomer is near the n-terminal region of the other (6). Such an arrangement, if confirmed, would allow the interaction of the PEST sequence with proteases. The sequence of LPL we describe is located in its carboxy-terminal region at the junction of exons 8 and 9. Except in the guinea pig, the sequence ends at a site of N-glycosylation (residue 359 in the human LPL). No other function has been attributed to this sequence (4). Moreover, no mutation of LPL causing enzyme deficiency has yet been reported for this PEST-rich region (7).

Proteins containing a PEST sequence may be sensitive to several proteolytic pathways (8). The Ca^{2+}-dependent proteases, calpains, are especially good candidates because apolipoprotein B, 3-hydroxy-3-methylglutaryl coenzyme A reductase and c-Fos contain PEST-rich sequences and are hydrolyzed by calpains (3,9,10). Moreover, the calpains I and II, and their specific inhibitor calpastatin, which is also Ca^{2+}-dependent, are distributed in a tissue-specific manner in accordance with the tissue-specific regulation of LPL. For instance, calpastatin is found in high concentration in the heart where LPL activity is the highest of all tissues (2,11). The content of calpastatin exceeds that of calpains I and II in rat heart and skeletal muscles, but not in adipose tissue (11). Calpains I and II are widely distributed cellular proteases active at μM and mM concentrations of Ca^{2+}, respectively. Higher concentrations of Ca^{2+} activate calpastatin (11). It is difficult to define the site at which Ca^{2+} might alter the degradation rate of LPL because Ca^{2+} metabolism is under the control of many organelles in addition to its transport across the plasma membrane (12). Moreover, Ca^{2+} can be sequestered at the surface of cell membranes by heparan sulfate proteoglycans (13). This is noteworthy because the proteoglycans that anchor LPL at the vascular endothelium modulate its secretion and lysosomal degradation and are effectively degraded by calpains *in vitro* (2,13).

Many agents that alter Ca^{2+} homeostasis have been shown to modify the activity of LPL. Indeed, the stimulatory effect of insulin on adipose LPL activity is mimicked or potentiated by the Ca^{2+} ionophore A23187, whereas blockade of transcription with cordicepin and alpha-amanitin do not alter it

*To whom correspondence should be addressed at Dept. Physiology, School of Medicine, Laval University, Québec (Qc), Canada, G1K 7P4.

Copyright © 1995 by AOCS Press

A

```
human       323 SGTESETHTNQAFEISLYGTVAESENIPFTLPEVSTNKTYSFLIYTEVDIGE 374
bovine      325 SGTESNTYTNQAFEISLYGTVAESENIPFTLPEVSTNKTYSFLLYTEVDIGE 376
guinea pig  323 SGTETTTYTNQAFEISLYGTVAESENIPFTLPEVSANNTYSFLIYTEVDIGE 374
mouse       323 SGTENGKQHNQAFEISLYGTVAESENIPFTLPEVSTNKTYSFLIYTEVDIGE 374
chicken     325 FGKTNVTKVDQPFLISLYGTLDESENIPFTLPEVSSNKTYSFLIYTEVDIGD 376
rat         323 SGTGNDLQDNQAFEISLYGTVAESENIPFTLPEVSTNKTYSFLIYTEVDIGE 374
```

B

	% PEST		% R,K,H		
	boxed sequence	whole LPL	flanking regions		whole LPL
human	(331-358) 46.4	(1-448) 25.0	(260-330) 26.8	(359-448) 21.1	15.8
bovine	(325-360) 50.0	(1-450) 24.2	(262-324) 28.6	(361-450) 18.9	15.3
guinea pig	(323-374) 45.6	(1-448) 25.9	(260-322) 27.0	(375-448) 24.0	15.8
mouse	(336-358) 52.2	(1-447) 23.7	(260-335) 26.3	(359-447) 20.2	15.9
chicken	(336-360) 48.0	(1-465) 22.8	(265-335) 26.8	(361-465) 18.1	15.1
rat	(336-358) 52.2	(1-447) 24.7	(260-335) 25.0	(359-447) 22.5	15.4

FIG. 1. The PEST-rich sequence of lipoprotein lipase (LPL) is found in the six species in which the protein has been cloned (panel A). Panel B shows that the frequency of the amino acids proline (P), glutamate (E), serine (S) and threonine (T) is high in the sequence in comparison with the whole LPL, whereas the flanking regions are enriched in the basic residues arginine (R), lysine (K) and histidine (H). Numbers refer to the amino acid position in the aligned sequences as published by Wang *et al.* (4) and Brault *et al.* (5).

(14). Moreover, LPL stimulation by insulin is abolished by depletion of cellular Ca^{2+} with EGTA (14). Thus, it may be that because insulin increases the cytosolic cellular Ca^{2+} content, the degradation rate of LPL in adipose tissue is decreased in the postprandial state (15,16). This mechanism does not exclude modulation of LPL at the transcriptional level, but Ca^{2+} could also inactivate the Ca^{2+}-dependent proteases acting on the PEST-rich sequence of LPL. This hypothesis is concordant with the fact that adipose LPL is chronically increased in obese subjects, who may also have a chronic increase in cytosolic Ca^{2+} concentration (1,17). In this situation, degradation of LPL might be blunted during the transition from the postprandial to the fasting state. Moreover, the absence of an acute insulin effect in adipose tissue of these subjects has been reported for both cytosolic Ca^{2+} and LPL activity (1,17). Such a mechanism of adipose LPL modulation by Ca^{2+}-dependent proteases is further supported by the observation that vanadate, which blocks the intracellular sequestration of Ca^{2+}, normalizes the lowered adipose LPL activity in streptozotocin-treated rats (18,19). In addition, epinephrine increases the Ca^{+} efflux from adipocytes and has been shown to increase the degradation of LPL (20,21).

It can also be argued that Ca^{2+}-dependent proteases modulate the activity of muscle LPL. Indeed, there is a large body of evidence suggesting that glucose uptake is Ca^{2+}-dependent (22). On the other hand, it has been shown *in vivo* that when glucose uptake is high, muscle LPL activity is low, such as during an insulin infusion (23). Thus, it can be hypothesized that Ca^{2+} is involved in the reciprocal modulation of LPL activity and glucose transport in muscles. That Ca^{2+} would be associated with divergent effects on adipose tissue and muscle LPL could stem from the tissue-specific relative abundance of calpains and their inhibitor calpastatin.

In summary, the PEST-rich sequence of LPL and a number of observations suggest a physiological role for Ca^{2+}-dependent proteases in the degradation of LPL. This means of controlling LPL activity could partly explain its tissue-specific regulation during the postprandial and fasting states.

André Boivin and Yves Deshaies*
Department of Physiology, School of Medicine
Laval University, Québec, G1K 7P4 Canada

REFERENCES

1. Eckel, R.H. (1989) *N. Engl. J. Med. 320*, 1060–1068.
2. Braun, J.E.A., and Severson, D.L. (1992) *Biochem. J. 287*, 337–347.
3. Dice, J.F. (1987) *FASEB J. 1*, 349–357.
4. Wang, C.-S., Hartsuck, J., and McConathy, W.J. (1992) *Biochim. Biophys. Acta 1123*, 1–17.
5. Brault, D., Noé, L., Etienne, J., Hamelin, J., Raisonnier, A., Souli, A., Chuat, J.C., Dugail, I., Quignard-Boulangé, A., Lavau, M., and Galibert, F. (1992) *Gene 121*, 237–246.
6. Wong, H., Davis, R.C., Thuren, T., Goers, J.W., Nikazy, J., Waite, M., and Schotz, M.C. (1994) *J. Biol. Chem. 269*, 10319–10323.
7. Lalouel, J.M., Wilson, D.E., and Iverius, P.H. (1992) *Curr. Opinion Lipidol. 3*, 86–95.
8. Rechsteiner, M. (1988) *Adv. Enzyme Regul. 29*, 135–151.
9. Sparks, J.D., and Sparks, C.E. (1993) *Curr. Opinion Lipidol. 4*, 177–186.
10. Hirai, S.-i., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991) *FEBS Lett. 287*, 57–61.
11. Murachi, T. (1989) *Biochem. Int. 18*, 263–294.
12. Pietrobon, D., Di Virgilio, F., and Pozzan, T. (1990) *Eur. J. Biochem. 193*, 599–622.
13. Suzuki, K., Shimizu, K., Hamamoto, T., Nakagawa, Y., Murachi, T., and Yamamuro, T. (1992) *Biochem. J. 285*, 857–862.
14. Vydelingum, N. (1983) *Am. J. Physiol. 245*, E121–E131.

15. Draznin, B., Kao, M., and Sussman, K.E. (1987) *Diabetes 36*, 174–178.
16. Doolittle, M.H., Ben-Zeev, O., Elovson, J., Martin, D., and Kirchgessner, T.G. (1990) *J. Biol. Chem. 265*, 4570–4577.
17. Draznin, B., Sussman, K.E., Eckel, R.H., Kao, M., Yost, T., and Sherman, N.A. (1988) *J. Clin. Invest. 82*, 1848–1852.
18. Rasgado-Flores, H., and Blaustein, M.P. (1987) *Am. J. Physiol. 252*, C588–C594.
19. Levy, E., and Bendayan, M. (1991) *Diabete & Metabolisme (Paris) 17*, 44–48.
20. Clarke, P.V., Kissebah, A.H., Hope-Gill, H., Vydelingum, N., Tulloch, B., and Fraser, T.R. (1975) *Eur. J. Clin. Invest. 5*, 351–358.
21. Ball, K.L., Speake, B.K., and Robinson, D.S. (1986) *Biochim. Biophys. Acta 877*, 399–405.
22. Aviv, A. (1992) *J. Am. Soc. Nephrol. 3*, 1049–1063.
23. Kiens, B., Lithell, H., Mikines, K.J., and Richter, E.A. (1989) *J. Clin. Invest. 84*, 1124–1129.

[Received September 28, 1994; accepted October 31, 1994]

ANNOUNCEMENTS

1995

June

2nd International Congress of the International Society for the Study of Fatty Acids and Lipids, June 7–10, National Institutes of Health, Bethesda, Maryland, USA. Information: A.P. Simopolous, ISSFAL, 2001 S St., N.W., Suite 530, Washington, DC 20009 USA.

18th Nordic Lipid Symposium, June 18–21, Reykjavik, Iceland. Information: G. Lambertsen, Lipidforum Secretariat, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-325724).

August

36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

EDITORIAL

A Southward Move for *Lipids*

This month *Lipids* celebrates its twenty-ninth birthday and moves its Editorial Office southward, albeit only 135 miles. Since its inception, the journal has had only four Editors and has firmly established itself as a premier source of new information on basic lipid research. Our founding Editor, A. Richard Baldwin, wrote a prescient editorial in the first issue of January 1966 in which he said: "The discovery of a possible relationship between the fat in the diet and cholesterol in the blood, and the development of new analytical techniques, have together breathed new life into what only a few years ago was considered a relatively exhausted and uninteresting field of research." Since that time, the awarding of a series of lipids-related Nobel prizes, the development of many new drugs targeting the metabolism or actions of lipid species, and the emergence of entire disciplines centered on lipid autacoids, hormones, protein modifiers, and gene regulators illustrate the enormous advance in our understanding of the importance of lipid molecules in biology.

The changes that led to the lipid renaissance have included not only technical improvements in chromatography, mass spectrometry, NMR, and other physical methods, but also the publication of definitive clinical trials verifying the importance of diet and cholesterol metabolism in human vascular disease. Epidemiologic evidence of the role of dietary fat in cancer, an appreciation of the importance of polyunsaturated fatty acids in human development, and of lipid oxidation in a number of disease processes has greatly increased research funding and activity in lipid-related fields during the past twenty-nine years. The regulation of gene expression by fatty acids, retinoids and other lipids, post-translational regulation of protein function by attachment of fatty acids and isoprenyl alcohols, and utilization of drugs altering lipid metabolism in experimental cancer therapy are only a few of the many "cutting edge" areas of lipid research.

Lipids has, indeed, adapted to the changing face of basic lipid research over nearly three decades. It is of interest that the first issue of *Lipids* included not only new analytical methods and lipid composition studies, but also papers presenting lipid–protein interactions, X-ray diffraction and mathematical modelling approaches. Studies published in recent issues, and those currently in press deal with molecular modelling based upon mathematical manipulation of X-ray crystallographical data, new mass spectrometric methods, and the molecular biology of lipid–protein interactions in both carrier proteins and enzymes. Also represented are works using a new generation of biophysical methods to provide novel perspectives on membrane structure and function. The realization that different families of polyunsaturated fatty acids may have different biological functions has led to numerous papers debating what makes particular fatty acids "essential" for a given species, and whether increasing the n-6 or n-3 fatty acid (or decreasing the *trans* fatty acid) content of the diet in industrial countries may provide health benefits. Thus, the papers published in *Lipids* now utilize a broad array of new and traditional techniques to explore areas of lipidology that are of great importance in medicine and biology. *Lipids* will continue to provide an expanding forum for basic and mechanistic work involving lipid research; as Dr. Baldwin described it in 1966, this includes studies on metabolism, nutrition, physiology, analytical methodology, natural products, and biomedical applications.

Many changes are taking place in the publishing field as well as in science. Our tax returns and NIH grants can now be submitted electronically, and contributors to *Lipids* are now usually submitting their final text on computer disk. Scientific journals are dealing with the problems of CD-ROM and on-line editions and their attendant copyright issues. *Lipids* will adapt to this new environment by bringing our readership the latest information as rapidly as an electronically enhanced peer-review system will allow, and a variety of new options are under study. To increase publication quality and efficiency, there will be a new font and some changes in article format. We will continue the practice of having timely Review Articles on a regular basis, and we welcome Letters-to-the-Editor on speculative or controversial topics. Portions of selected issues may be devoted to presenting peer-reviewed papers from significant lipids meetings, and our Announcements page will include mention of meetings and other special items pertinent to workers in lipid research. Although the field of lipid research was "considered a relatively exhausted and unimportant" one in 1960, our perspective 35 years later is that it will only continue to expand in the decades ahead and that we will make every effort to have *Lipids* serve as an integral part of that expansion.

The guiding philosophy of *Lipids* has remained the same throughout the tenure of my four predecessors. As stated by Dr. Wolfgang Baumann in his 1986 editorial, "a journal's success is measured by its appeal to contributors and readers," and "the editorial philosophy of *Lipids* will be guided by the desire to reach beyond past accomplishments." Last month's issue included a tribute to Dr. Baumann's extraordinary efforts to serve the journal and the scientific community, and

these efforts have continued in his assuring a smooth editorial transition. I am especially grateful for his many words of advice and encouragement. To continue the level of excellence achieved by my predecessors, I am also fortunate to have the support of Mary Lane and her staff at AOCS Press, our distinguished group of Associate Editors and Editorial Advisory Board members, and the continued cooperation of our excellent reviewers. In an era of rapid change on many fronts, it is essential that we receive the suggestions and perspectives of many lipid researchers in addition to their other contributions, and these are always welcome. The importance of any journal, after all, lies in its usefulness and responsiveness to the scientific community it serves.

Howard R. Knapp
Editor-in-Chief

Hepoxilins: A Review on Their Enzymatic Formation, Metabolism and Chemical Synthesis

C.R. Pace-Asciak[a,b,*], Denis Reynaud[a] and Peter M. Demin[a]

[a]Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8 Canada and [b]Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario, M5S 1A8 Canada

ABSTRACT: This article reviews published evidence describing the enzymatic and nonenzymatic formation and the routes of metabolism of the hepoxilins. Also treated are the major approaches used for the chemical synthesis of these compounds and for some of their analogs.
Lipids 30, 107–114 (1995).

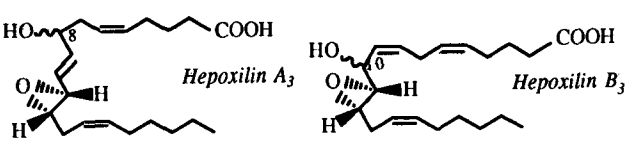
The hepoxilin pathway was discovered over a decade ago (1–5), and considerable research since has shown a variety of interesting biological activities, the basis of which appears to relate to the release of intracellular calcium (6) and to the opening of potassium channels (7,8). Hepoxilins appear to have pro-inflammatory actions in the skin (9–11), but appear to inhibit the actions of inflammatory agents in the human neutrophil (12). Hepoxilin receptors have recently been identified which are coupled to G-proteins (13). For reviews on the cellular actions of the hepoxilins, the reader is referred to recent reviews (14,15). This brief article reviews evidence for the enzymatic formation and metabolism of the hepoxilins. Also addressed is a review of the diverse approaches used for the chemical synthesis of these compounds, as well as several stable analogs which also possess biological activity.

NOMENCLATURE

Hepoxilins are monohydroxy-epoxide derivatives of arachidonic acid formed from the initial unstable product of 12-lipoxygenase, 12(*S*)-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid [12(*S*)-HPETE] (2). The term hepoxilin was coined to combine structure (Hydroxy EPOXide) with their first reported biological activity (release of InsuLIN) (4). Two hepoxilins have been isolated, i.e., 8(*S/R*)-hydroxy-11(*S*),12(*S*)-*trans*-epoxyeicosa-5*Z*,9*E*,14*Z*-trienoic acid (hepoxilin A_3, HxA_3) and 10(*S/R*)-hydroxy-11(*S*),12(*S*)-*trans*-epoxyeicosa-5*Z*,8*Z*,14*Z*-trienoic acid (hepoxilin B_3, HxB_3). This terminology is consistent with that used in the prostaglandin field where the subscript denotes the number of double bonds in the molecule. The "A" and "B" designation relates to the carbon position of the hydroxyl group; A refers to a hydroxyl group at C8, B denotes one at C10. As the epoxide group is relatively unstable biologically with further metabolic reactions taking place (see section on metabolism), the designated metabolites are referred to the initial hepoxilin, e.g., the glutathione conjugate is referred as hepoxilin A_3-C, by analogy with the leukotriene system of naming these conjugates. Hydrolysis of the epoxide generates trihydroxy metabolites, 8(*S/R*),11(*R*),12(*S*)-trihydroxy-eicosa-5*Z*,9*E*,14*Z*-trienoic acid (trioxilin A_3, $TrXA_3$) and 10(*S/R*),11(*R*),12(*R*)-trihydroxy-eicosa-5*Z*,8*Z*,14-trienoic acid (trioxilin B_3, $TrXB_3$). As mentioned above, these products are derived from arachidonic acid. Similar products have been isolated from eicosapentaenoic acid (16).

Scheme 1 describes the structures of HxA_3 and HxB_3.

Isolation. Indirect evidence for the formation of hepoxilins was first obtained by Jones *et al.* in 1978 (17) when a trihydroxy metabolite of arachidonic acid was reported formed by washed human platelets. That product, 10,11,12-trihydroxy-eicosatrienoic acid, was proposed to be derived from a 10-hydroxy-11,12-epoxide intermediate. Subsequently, these authors isolated a hydroxy-epoxide (18) which was later called hepoxilin B_3 (14). Bryant and Bailey (19) later reported the structure of two trihydroxy metabolites formed by human platelets bearing hydroxyl groups at C8, C11 and C12, and C8, C9 and C12 also proposing their formation through an epoxide intermediate. It is now known that these compounds arise from hepoxilin A_3 during its stereo-uncontrolled acid-catalyzed hydrolysis (20). Interestingly, formation of these metabolites was stimulated by glucose in the incubation



SCHEME 1

*To whom correspondence should be addressed at the Research Institute, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, M5G 1X8 Canada.

Abbreviations: GST, glutathione S-transferase; hepoxilin A_3, HxA_3, 8(*S/R*)-hydroxy-11(*S*),12(*S*)-*trans*-epoxy-eicosa-5*Z*,9*E*,14*Z*-trienoic acid; hepoxilin A_3-C, HxA_3-C, 8(*S/R*),12(*S*)-dihydroxy-11(*R*)-glutathionyl-eicosa-5*Z*,9*E*,14*Z*-trienoic acid; hepoxilin B_3, HxB_3, 10(*S/R*)-hydroxy-11(*S*),12(*S*)-*trans*-epoxy-eicosa-5*Z*,8*Z*,14*Z*-trienoic acid; 12-HETE, 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; 12(*S*)-HPETE, 12(*S*)-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; TCPO, trichloropropene oxide; trioxilin A_3, $TrXA_3$, 8(*S/R*),11(*R*),12(*S*)-trihydroxy-eicosa-5*Z*,9*E*,14*Z*-trienoic acid; trioxilin B_3, $TrXB_3$, 10(*S/R*),11(*R*),12(*R*)-trihydroxy-eicosa-5*Z*,8*Z*,14*Z*-trienoic acid.

Copyright © 1995 by AOCS Press

medium (21). These compounds were later found to be formed in rat lung (1). Pace-Asciak *et al.* isolated from rat lung the two hydroxy-epoxide intermediates that yielded the above mentioned trihydroxy metabolites, and their structures were determined to be the isomeric 8-hydroxy and 10-hydroxy-11,12-epoxide derivatives of arachidonic acid (2), later called hepoxilins A_3 and B_3 (4). It was also recognized that the epoxide ring was acid labile, providing a reason why previous reports isolated only the trihydroxy products. The epoxide group of each epimer (8*S* or 8*R*) of hepoxilin A_3 is also subject to enzymatic hydrolysis to yield one trihydroxy product, 8(*S* or *R*),11(*R*),12(*S*)-trihydroxyeicosa-5*Z*,9*E*,14*Z*-trienoic acid. As biological testing on pancreatic islets demonstrated that the isolated hydroxy-epoxides potentiated the glucose-evoked release of insulin (4), the products were named "hepoxilins" to combine structural features with their insulin secretory properties (4).

Formation of the hepoxilins has been reported in human platelets and lung, liver, pancreas, brain, aorta and pineal gland of the rat (for review see Refs. 14 and 15). They are also formed by the *Aplysia* brain (22), and a report by Moghaddam *et al.* (23) indicates that they are also formed in tropical marine algae. Hepoxilin A_3 has been identified as the endogenous factor regulating volume decrease in human platelets (7,8). Trioxilin B_4 has been identified as the hatching factor in barnacles (24), whereas trioxilin A_4 was isolated from fish gills (25).

BIOSYNTHESIS

Hepoxilins are formed from 12(*S*)-HPETE (2). Early work with 12(*S*)-HPETE in which the hydroperoxide group was labeled with ^{18}oxygen, derived from experiments involving the reaction of platelet 12(*S*)-lipoxygenase with d_8-arachidonic acid, demonstrated the complete retention of ^{18}oxygen in both the hydroxyl and epoxide moieties in the resulting hepoxilins when the 12-HPETE was incubated with a lung homogenate (26). These experiments demonstrated that hepoxilins are formed from 12-HPETE and that the oxygen atoms in hepoxilins are derived through an intramolecular isomerization involving an intermediate which excludes external oxygen from the incubation medium. Such an internalized isomerization is typical of enzymatic catalysis, except that the retention of oxygen from the hydroperoxide is observed both with a rat lung preparation as well as with hemin or hemoglobin as catalyst (5), indicative of a pseudo-enzymatic reaction (26). This type of transformation suggested that hepoxilin synthesis may be nonenzymatic, even though it typified an enzymatic-type transformation from 12-HPETE. Only recently, through use of rapid nonacidic extraction methods and derivatization into stable fluorescent ester derivatives, has unequivocal information been obtained to demonstrate the presence of a heat-inactivatable protein in the rat pineal gland that forms hepoxilin A_3 (27). In these studies, a racemic mixture of 12(*S*)- and 12(*R*)-HPETE, formed through the light-induced autoxidation of arachidonic acid, was incubated with intact (slices of) pineal glands, and the products were characterized by high-performance liquid chromatography (HPLC) after appropriate derivatization (and stabilization) into fluorescent ester derivatives. Several important findings were observed in these experiments. First, hepoxilin A_3 was the major hepoxilin formed with the intact tissue, little hepoxilin B_3 was found. In contrast, when hemin was used as catalyst in the absence of the tissue, both hepoxilin A_3 and B_3 were formed from the 12-HPETE racemic mixture, in approximately equal amounts. Boiling of the pineal gland abolished all hepoxilin formation, whereas boiling of hemin did not affect hepoxilin formation. A similar conversion of arachidonic acid by the pineal gland into mostly hepoxilin A_3 of the native 11(*S*),12(*S*)-configuration of the epoxide is observed, indicating the presence of 12(*S*)-lipoxygenase in the pineal gland (28,29), which is coupled to hepoxilin A_3 synthase. Arachidonic acid is not converted into products when incubated with hemin. As with 12-HPETE, boiling of the pineal tissue abolished all enzymatic conversion of arachidonic acid (see Fig. 1). Second, when 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE) was purified from both the fresh pineal experiments and the

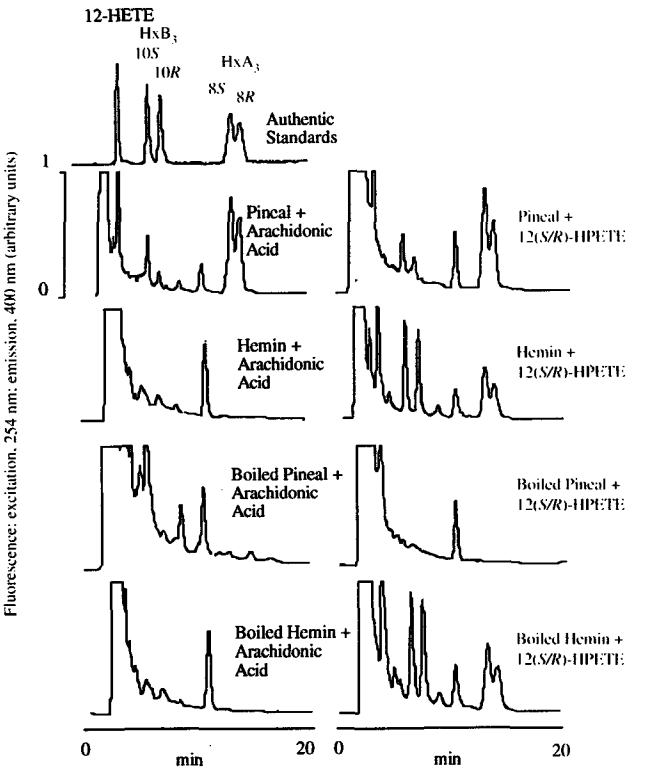
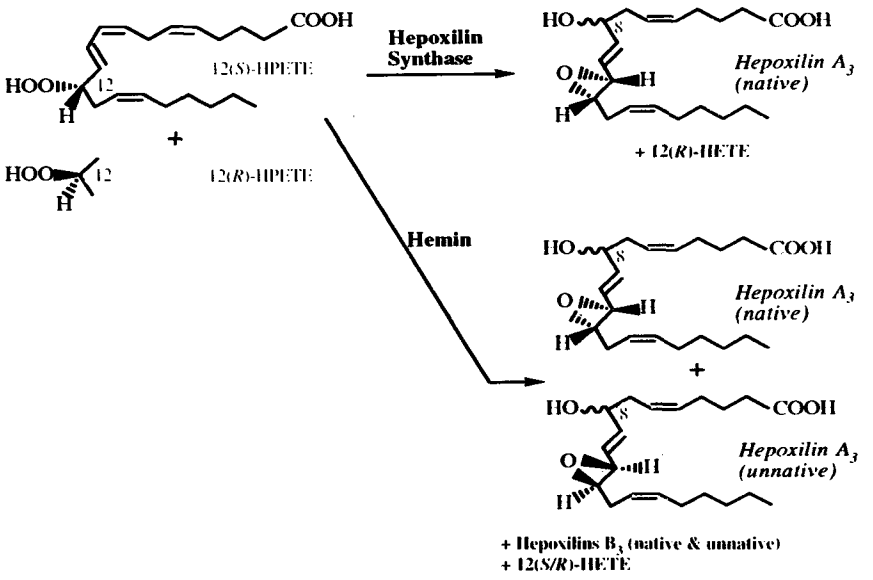


FIG. 1. Experiments to show the enzymatic nature of hepoxilin synthesis in the pineal gland in contrast to the nonenzymatic formation by hemin. Note the different ratio of hepoxilin A_3 (HxA_3) vs. hepoxilin B_3 (HxB_3) formed by the pineal in contrast to hemin catalysis. Also note the total abolition of hepoxilin synthesis by boiling the pineal [both from arachidonic acid as well as 12-HPETE (hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid) substrates—second panels from bottom] in contrast to the lack of effect during hemin catalysis (bottom right panel). These experiments provide strong support for an enzymatic pathway in the formation of HxA_3. HETE, hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid.



SCHEME 2

hemin experiments and analyzed by chiral phase HPLC, it was found that the 12(*S*)-HETE was absent in the pineal gland experiments, only 12(*R*)-HETE was present because all the 12(*S*)-HPETE in the epimeric substrate was used up in the formation of hepoxilin A_3. In contrast, in the hemin experiments both 12(*S*)- and 12(*R*)-HETEs were found in the same epimeric proportion as the starting HPETE because hemin was not substrate selective.

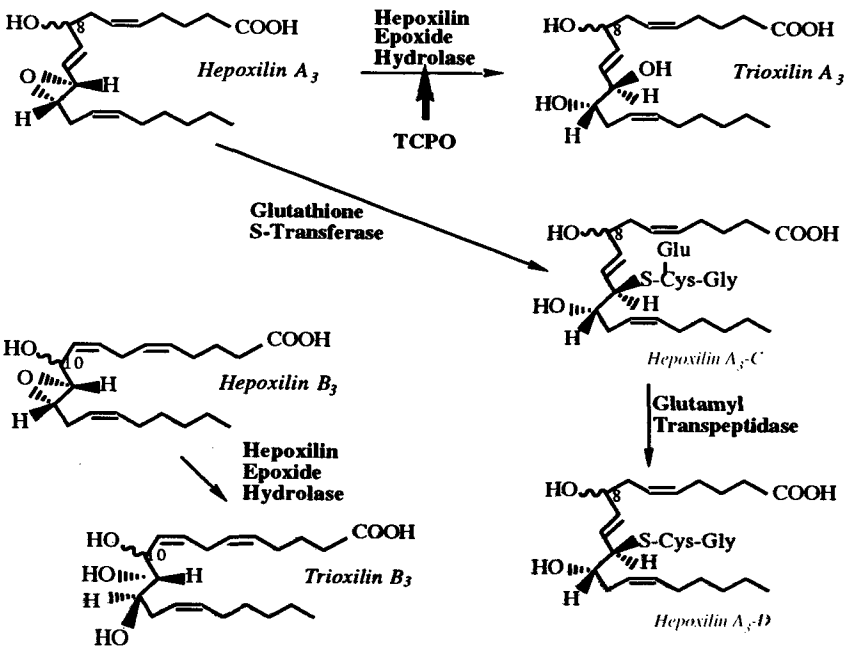
These experiments indicate a stereoselection of 12(*S*)-HPETE by the pineal gland, but not by hemin, in the formation of hepoxilin A_3. Third, chiral phase analysis of hepoxilin A_3 formed by the pineal gland confirmed the natural *S,S-trans* configuration of the epoxide group, while both the (11*S*,12*S*)- and the (11*R*,12*R*)-*trans* hepoxilins A_3 were formed during hemin catalysis, further demonstrating the nonselectivity of hemin. The nonenzymatic isomerization of HPETEs in the linoleic acid series has been previously observed (30–32). These important new findings are summarized in Scheme 2.

These experiments demonstrate that hepoxilin A_3 formation is carried out by a specific heat-labile protein (hepoxilin A_3 synthase) which catalyzes the transformation of 12(*S*)-HPETE almost exclusively into the 8(*S*)- and 8(*R*)-epimers of hepoxilin A_3 with only minor amounts of hepoxilin B_3 formed. It seems that hepoxilin B_3 may not be an enzymatic product as it is formed only to a small extent by the intact pineal gland and may in fact be due to catalysis by some hemoprotein. Hepoxilin A_3 synthase appears to be a cytosolic protein whose characterization is in progress. Transformation of 12(*S*)-HPETE by this protein does not seem to require any cofactors.

METABOLISM

Two pathways have been described for the metabolism of hepoxilin A_3 (33,34). Hepoxilin B_3 is more chemically stable than hepoxilin A_3, although it is also metabolized (2). Scheme 3 describes the two metabolic pathways for hepoxilin A_3 metabolism. The first pathway involves solvolysis of the epoxide by water, the new hydroxyl group entering at C11 with inversion of configuration, i.e., (11*R*). The newly formed trihydroxy metabolite is termed trioxilin A_3. The responsible enzyme appears to be selective for the hepoxilin structure as the partially purified protein appears to prefer hepoxilin A_3 as substrate in comparison to leukotriene A_4 or styrene oxide (35). In contrast, the acid-catalyzed hydrolysis of hepoxilin A_3 leads to a mixture of trioxilin epimers with hydroxyl groups at C8, C11 and C12, as well as C8, C9 and C12 (20). In contrast to the metabolism of hepoxilin A_3, hepoxilin B_3 is metabolized with epoxide ring opening through an attack of the hydroxyl group at C12, as well as with inversion of configuration, i.e., (12*R*) (2). The second pathway described for the metabolism of hepoxilin A_3 involves the addition of glutathione at C11 of the epoxide moiety to afford an 11(*R*)-glutathionyl-8(*R/S*),12(*S*)-dihydroxy metabolite of hepoxilin A_3 (hepoxilin A_3-C, HxA_3-C) (33,36). The reaction is carried out by a glutathione S-transferase (GST). Work with purified isozymes of rat liver GST has shown that the reaction is best carried out by a homodimer containing the Y_{b2} subunit while isozymes containing the Y_a or the Y_c subunits showed marginal activity (37). The same subunit converts leukotriene A_4 into C_4 (38). In contrast, the conversion of the prostaglandin endoperoxide PGH_2 into PGE_2 and PGF_{2a} requires the Y_a subunit (39). Chemical opening of the epoxide by glutathione gives by analogy with chemical hydrolysis, a mixture of 11(*R*)- and 9(*S/R*)-glutathionyl adducts (40).

Hepoxilin epoxide hydrolase is inhibited by trichloropropene oxide (TCPO), a general epoxide hydrolase inhibitor (36). In the presence of TCPO, HxA_3 metabolism is shunted toward glutathione conjugation with the formation of HxA_3-C (36). HxA_3-C formation has been shown to occur in rat aorta (41) and in rat brain (36). HxA_3-C shares with its precursor



SCHEME 3

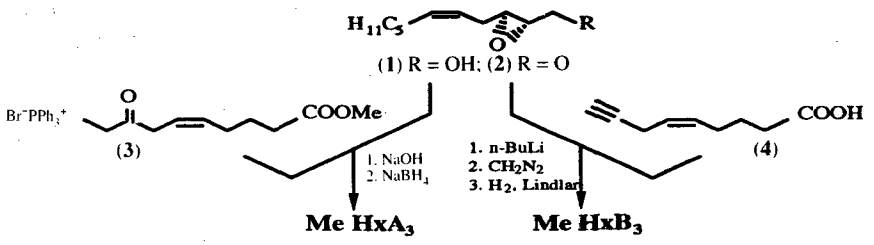
some of the biological activity in some systems. Specifically, HxA_3-C is capable of potentiating the norepinephrine-induced contraction of the rat aorta (42), portal vein (42) and the guinea pig trachea (43), and the bradykinin-induced vascular permeability in rat skin (10). The compound also shows an enhancing action of electrical potentials in rat hippocampal CA1 neurons (44,45), specifically the resting membrane potential, post spike train after-hyperpolarization and the inhibitory postsynaptic potential but not the excitatory postsynaptic potential. The former two actions of HxA_3-C are epimer-specific, with the (8*R*) epimer showing activity exclusively in the vascular reactivity assay, whereas the (8*S*) epimer is the active species on vascular permeability. These epimer-specific actions suggest receptor-mediation. In fact HxA_3-specific binding proteins have been described in human neutrophils which appear to be intracellular in localization, and mediated through G-protein interaction (13).

CHEMICAL SYNTHESIS OF HEPOXILINS AND SOME STABLE ANALOGS

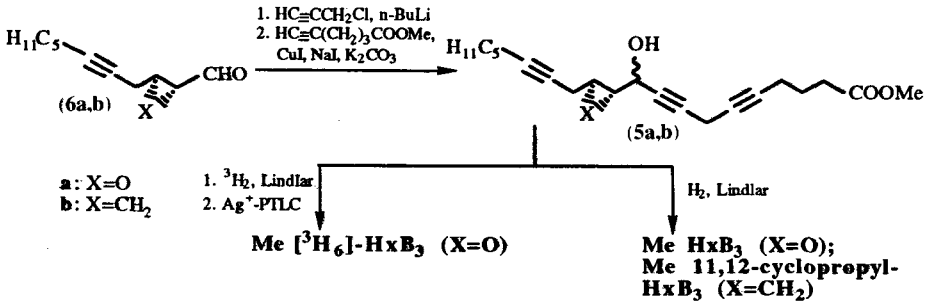
Several different approaches have been described for the chemical synthesis of the hepoxilins. These different approaches will be looked at through the key intermediates used by different groups from which the hepoxilin structure was generated.

The first total synthesis of HxB_3 and HxA_3 was developed by Corey *et al.* (46,47) on the basis of methods used for the synthesis of other acyclic eicosanoids. Both types of hepoxilins were obtained using a common epoxy alcohol **1**, readily available *via* Sharpless asymmetric epoxidation of the corresponding (*E*)-allylic alcohol. After oxidation of **1** into the aldehyde **2**, it was condensed with either **3** as its triphenylphosphonium salt or lithium derivative of terminal acetylene **4** creating γ- or α-hydroxy epoxy fragments of HxA_3 and HxB_3 (Scheme 4).

The C8- and C10-epimeric mixtures of HxA_3 and HxB_3 obtained were finally separated as individual diastereomers, and their chromatographic and spectral properties were in agreement with compounds isolated from natural sources. The use of acetylenic fragments has led to the synthesis of deuterium- or tritium-labelled analogs of hepoxilins. The triacetylenic analog of HxB_3 **5a** allowed the preparation of hepoxilins (A and B) with high specific activity (48). The relatively unstable triacetylene **5a** was synthesized by Vasiljeva and co-workers (49) in high yield through the condensation



SCHEME 4



SCHEME 5

of the acetylenic epoxyaldehyde **6a** with the Li-derivative of propargylchloride and methyl 5-hexynoate (49). Partial catalytic hydrogenation of **5a** in Lindlar conditions secured high selectivity of the three (*Z*)-double bonds (Scheme 5). Starting from the cyclopropyl aldehyde **6b**, cyclopropyl analogs of HxB_3 have been prepared (50).

Chabert *et al.* (51) developed a new approach to create the γ-hydroxy epoxy fragment of HxA_3 using arsonium ylides to lead to the formation of the epoxide. A stable readily available arsonium salt **7** was condensed with γ-hydroxy vinylic aldehyde **8** to give the (±)-methyl ester of HxA_3 (Scheme 6).

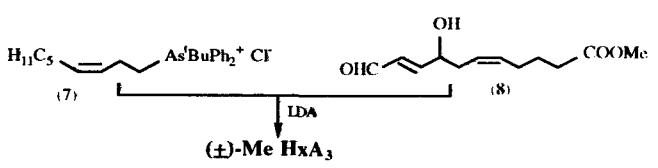
A number of approaches to optically active HxB_3 employed available natural chirones. Wu and Wu (52,53) used isopropilydene derivatives of *vic*-triols **9** and **10** obtained from (−)-D-tartaric acid and D-mannose, to prepare HxB_3 (52,53). Wittig condensations of **9** and **10** with corresponding synthons led to the protected 10,11,12-triols; C10 individual epimers of HxB_3 were obtained by subsequent tosylation/deprotection/epoxide ring closure (Scheme 7).

These synthetic schemes permitted the preparation of individual optical isomers of $TrxB_3$, the trihydroxy metabolite, which might be useful in studies investigating the naturally occurring products of hydrolysis of HxB_3. Hence, in another synthesis (−)-quinic acid was utilized as a source of chirality to prepare the triol **14** with differentiated hydroxyl groups (54). This approach afforded all possible four stereoisomers of HxB_3 derived from the *trans*-epoxide as well as $TrxB_3$, including the unnatural (11*R*,12*R*)-enantiomers of hepoxilins that would be formed from (12*R*)-HPETE (Scheme 8).

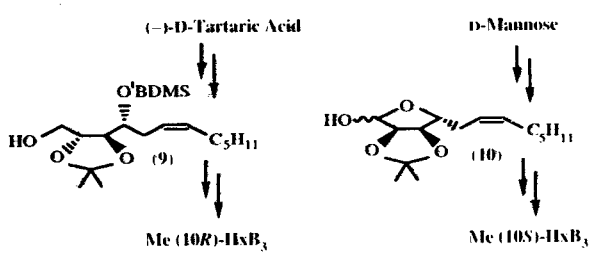
Transformations of hepoxilins. Because HxB_3 is more chemically stable than HxA_3, and as both are allylic isomers, methods for the interconversion of HxB_3 into HxA_3 through allylic transposition of the hydroxyl group have been investigated (Scheme 9). Demin *et al.* (55) developed an approach to HxA_3 synthesis from HxB_3 based on the solvolysis of unstable HxB_3 mesylates leading to a mixture of HxA_3 and B_3. This allylic rearrangement involves an S_N2' mechanism with a moderate (1.6:1) preference of C8-epimer of HxA_3 arising from the *syn*-attack of H_2O as a neutral nucleophile to afford the C8-epimer with an *anti* configuration of the C8 hydroxyl group. The C8-configuration of the individual epimers of HxA_3 was assigned according to this mechanism. The configuration of the carbinolic centres of HxA_3 was also established on the basis of products of ozonolysis of HxA_3 acetates (40).

Stable cyclopropyl analogs of HxB_3 were similarly transformed to cyclopropyl-HxA_3 as their acetates *via* standard Pd (II)-promoted mechanism (50). Lumin *et al.* (56) improved this Pd (II)-dependent process and developed a Pd (II)-directed allylic Mitsunobu displacement to rearrange protected triol **12** into its C8-isomer including Pd(II) interception of an oxyphosphonium intermediate. Subsequent deprotection/ring closure led to (8*R*)-HxA_3 in high yield and diastereoselectivity. Its (8*S*)-epimer was obtained in a similar manner (Scheme 10).

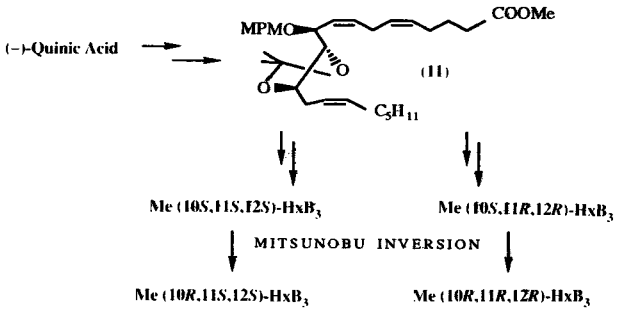
It was also shown that the classic Mitsunobu inversion of carbinolic centers of hepoxilins is accompanied by allylic rearrangement to some degree (Scheme 11). This was used in



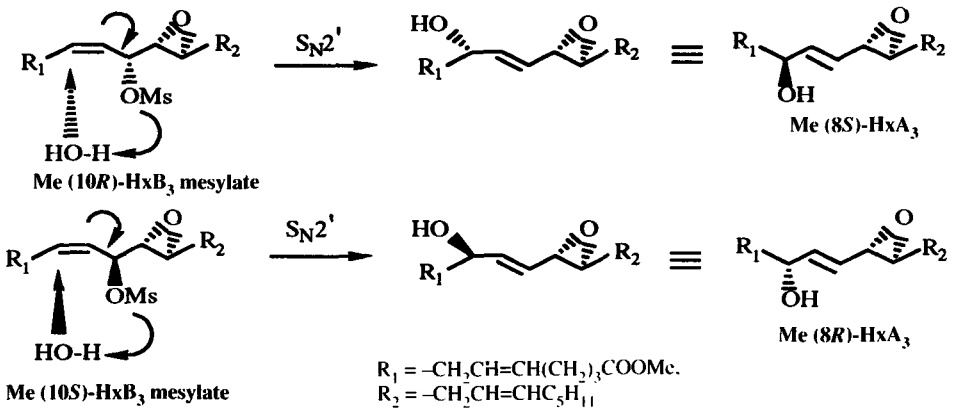
SCHEME 6



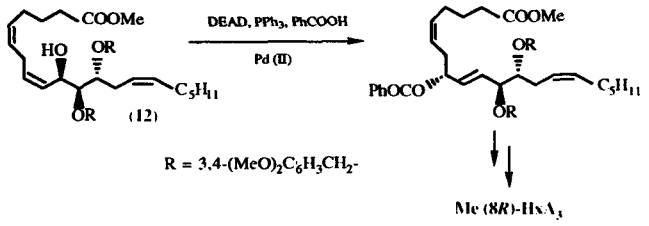
SCHEME 7



SCHEME 8



SCHEME 9



SCHEME 10

the preparation of tritium-labeled HxA_3, using labeled HxB_3 as a starting material. Very mild conditions permitted the handling of highly radioactive material (48). Employing this method, all possible regio- and stereo-isomers of hepoxilins were prepared (57).

By analogy with peptido-leukotrienes, chemical opening of the epoxide in hepoxilins by *N*-trifluoroacetate of glutathione was achieved, and glutathione adducts of HxA_3 (40) and HxB_3 (54) were synthesized. In case of HxA_3, C11 (*R*) thioether conjugate, as well as two C9 (*R/S*) thioether conjugates, were obtained as a separable mixture, whereas for HxB_3 a sole product of glutathione attack at C12 was isolated (Scheme 12).

CONCLUSIONS

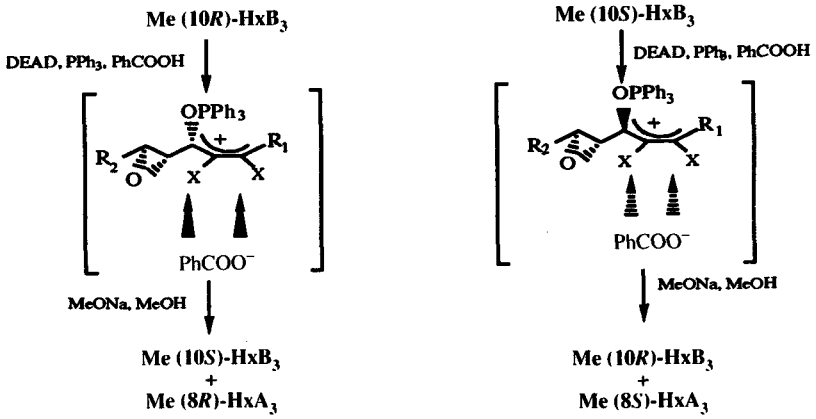
This review is intended to summarize literature available to date on the biochemistry and chemistry of the hepoxilins, a rapidly emerging area of eicosanoid research. This review may prove helpful in view of the recent demonstration of the existence of an enzyme system which forms HxA_3 specifically from 12(*S*)-HPETE, and in view of the demonstration of receptor-mediated actions. Stable analogs of the hepoxilins may prove useful in furthering this research.

ACKNOWLEDGMENTS

The financial support of the Canadian MRC and of ZymoGenetics Inc. is acknowledged for the preparation of this article and for the studies originating from the author's laboratory reported in this review. The collaboration of numerous investigators in the studies reported herein is also acknowledged.

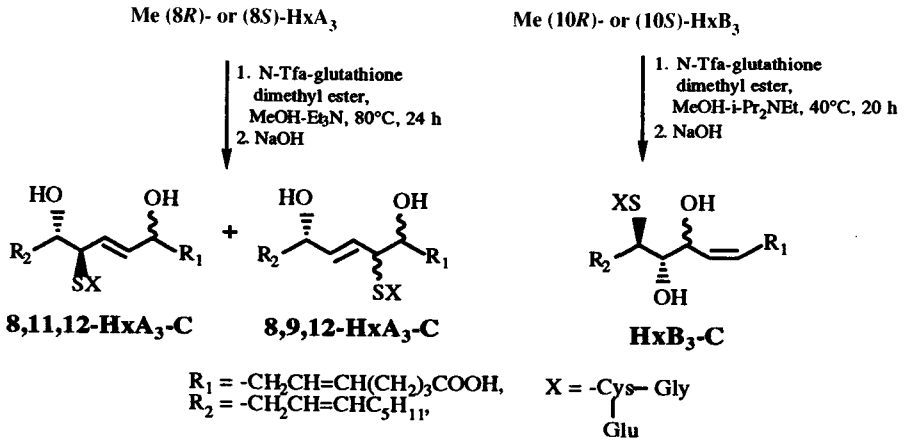
REFERENCES

1. Pace-Asciak, C.R., Mizuno, K. and Yamamoto, S. (1982) *Biochim. Biophys. Acta 712*, 142–145.
2. Pace-Asciak, C.R., Granström, E., and Samuelsson, B. (1983) *J. Biol. Chem. 258*, 6835–6840.



R_1 = -CH_2CX=$CX(CH_2)_3COOMe$, R_2 = -CH_2CX=CXC_5H_{11}, X = ^1H, ^3H

SCHEME 11



SCHEME 12

3. Pace-Asciak, C.R., Mizuno, K., and Yamamoto, S. (1983) *Prostaglandins 25*, 79–84.
4. Pace-Asciak, C.R., and Martin, J.M. (1984) *Prostagl. Leukotrienes and Med. 16*, 173–180.
5. Pace-Asciak, C.R. (1984) *Biochim. Biophys. Acta 793*, 485–488.
6. Dho, S., Grinstein, S., Corey, E.J., Su, W.G., and Pace-Asciak, C. R. (1990) *Biochem. J. 266*, 63–68.
7. Belardetti, F., Campbell, W.B., Falck, J.R., Demontis, G., and Rosolowsky, M. (1989) *Neuron 3*, 497–505.
8. Margalit, A., Sofer, Y., Grossman, S., Reynaud, D., Pace-Asciak, C.R., and Livne, A. (1993) *Proc. Natl. Acad. Sci. (USA) 90*, 2589–2592.
9. Laneuville, O., Corey, E.J., and Pace-Asciak, C.R. (1991) in *Prostaglandins, Leukotrienes & Lipoxins, XIth, Washington International Spring Symposium* (Bailey J.M., ed.). Abstract #155, Plenum Press, New York.
10. Laneuville, O., Corey, E.J., Couture, R., and Pace-Asciak, C.R. (1991) *Eicosanoids 4*, 95–97.
11. Laneuville, O., and Pace-Asciak, C.R. (1991) in *Prostaglandins, Leukotrienes, Lipoxins and PAF* (Bailey, J.M., ed.), pp. 335–338, Plenum Press, New York.
12. Laneuville, O., Reynaud, D., Grinstein, S., Nigam, S., and Pace-Asciak, C.R. (1993) *Biochem. J. 295*, 393–397.
13. Reynaud, D., Demin, P., and Pace-Asciak, C.R. (1995) in *Mediators in the Cardiovascular System—Regional Ischemia* (Schrör, K., and Pace-Asciak, C.R., eds.), *Agents and Actions Supplements,* Vol. 45, pp. 291–296, Birkhauser Verlag, Basel.
14. Pace-Asciak, C.R. (1994) *Biochim. Biophys. Acta 1215*, 1–8.
15. Pace-Asciak, C.R. (1993) *Gen. Pharmac. 24*, 805–810.
16. Pace-Asciak, C.R. (1986) *Prostagl. Leukotrienes. Med. 22*, 1–9.
17. Jones, R.L., Kerry, P.J., Poyser, N.L., Walker, I.C., and Wilson, N.H. (1978) *Prostaglandins 16*, 583–590.
18. Walker, I.C., Jones, R.L., and Wilson, N.H. (1979) *Prostaglandins 18*, 173–178.
19. Bryant, R.W., and Bailey, J.M. (1979) *Prostaglandins 17*, 9–18.
20. Demin, P.M., Vasiljeva, L.L., Myagkova, G.I., and Pivnitsky, K.K. (1991) *Bioorg. Khim. 17*, 1133–1140.
21. Bryant, R.W., and Bailey, J.M. (1980) *Adv. Prostagl. Thromb. Res. 6*, 95–100.
22. Piomelli, D., Shapiro, E., Zipkin, R., Schwartz, J.H., and Feinmark, S.J. (1989) *Proc. Natl. Acad. Sci. (USA) 86*, 1721–1725.
23. Moghaddam, M.F., Gerwick, W.H., and Ballantine, D.L. (1990) *J. Biol. Chem. 265*, 6126–6130.
24. Holland, D.L., East, J., Gibson, K.H., Clayton, E., and Oldfield, A. (1985) *Prostaglandins 29*, 1021–1029.
25. German, B., Bruckner, G., and Kinsella, J. (1983) *Prostaglandins 26*, 207–210.
26. Pace-Asciak, C.R. (1984) *J. Biol. Chem. 259*, 8332–8337.
27. Reynaud, D., Demin, P., and Pace-Asciak, C.R. (1994) *J. Biol. Chem. 269*, 23976–23980.
28. Hada, T., Hagiya, H., Suzuki, H., Arakawa, T., Nakamura, M., Matsuda, S., Yoshimoto, T., Yamamoto, S., Azekawa, T., Morita, Y., Ishimura, K., and Kim, H.-Y. (1994) *Biochim. Biophys. Acta 1211*, 221–228.
29. Yoshimoto, T., Hagiya, H., and Yamamoto, S. (1993) in *International Symposium on Molecular Biology of the Arachidonate Cascade,* Kyoto, Japan, December 5–7, 1993, Abs. p. 55.
30. Hamberg, M. (1975) *Lipids 10*, 87–92.
31. Graveland, A. (1970) *Biochem. Biophys. Res. Commun. 41*, 427–434.
32. Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1976) *Eur. J. Biochem. 62*, 33–36.
33. Pace-Asciak, C.R., Laneuville, O., Chang, M., Reddy, C.C., Su, W.-G., and Corey, E.J. (1989) *Biochem. Biophys. Res. Commun. 163*, 1230–1234.
34. Pace-Asciak, C.R., Klein, J., and Spielberg, S.P. (1986) *Biochim. Biophys. Acta 875*, 406–409.
35. Pace-Asciak, C.R., and Lee, W.-S. (1989) *J. Biol. Chem. 264*, 9310–9313.
36. Pace-Asciak, C.R., Laneuville, O., Su, W.-G., Corey, E.J., Gurevich, N., Wu, P., and Carlen, P L. (1990) *Proc. Natl. Acad. Sci. (USA) 87*, 3037–3041.
37. Laneuville, O., Chang, M., Reddy, C.C., Corey, E.J., and Pace-Asciak, C.R. (1990) *J. Biol. Chem. 265*, 21415–21418.
38. Mannervik, B., Jensson, H., Alin, P., Örning, L., and Hammarström, S. (1984) *FEBS Lett. 174*, 289–293.
39. Chang, M., Hong, Y., Burgess, J.R., Tu, C.-P.D., and Reddy, C.C. (1987) *Arch. Biochem. Biophys. 259*, 548–557.
40. Corey, E.J., and Su, W.-G. (1990) *Tetrahedron Lett. 31*, 2113–2116.
41. Laneuville, O., Corey, E.J., Couture, R., and Pace-Asciak, C.R. (1991) *Biochim. Biophys. Acta 1084*, 60–68.
42. Laneuville, O., Couture, R., and Pace-Asciak, C.R. (1992) *Br. J. Pharmacol. 105*, 297–304.
43. Laneuville, O., Couture, R., and Pace-Asciak, C.R. (1992) *Br. J. Pharmacol. 107*, 808–812.
44. Carlen, P.L., Gurevich, N., Wu, P.H., Su, W.-G., Corey, E.J., and Pace-Asciak, C.R. (1989) *Brain Res. 497*, 171–176.
45. Carlen, P.L., Gurevich, N., Zhang, L., Wu, P.H., Reynaud, D., and Pace-Asciak, C.R. (1994) *Neuroscience 58*, 493–502.
46. Corey, E.J., and Su, W.-G. (1984) *Tetrahedron Lett. 25*, 5119–5122.
47. Corey, E.J., Kang, J., Laguzza, B C., and Jones, R.L. (1983) *Tetrahedron Lett. 24*, 4913–4916.

48. Demin, P.M., Pivnitsky, K.K., Vasiljeva, L.L., and Pace-Asciak, C.R. (1994) *J. Labelled Compounds and Radiopharmaceuticals 34*, 221–230.
49. Vasiljeva, L.L., Manukina, T.A., Demin, P.M., Lapitskaja, M.A., and Pivnitsky, K.K. (1993) *Tetrahedron 49*, 4099–4106.
50. Demin, P.M., and Pace-Asciak, C.R. (1993) *Tetrahedron Lett. 34*, 4305–4308.
51. Chabert, P., Mioskowski, C., and Falck, J.R. (1989) *Tetrahedron Lett. 30*, 2545–2548.
52. Wu, W.L., and Wu, Y.L. (1993) *J. Org. Chem. 58*, 2760–2762.
53. Wu, W.L., and Wu, Y.L. (1992) *J. Chem. Soc. Perkin Trans. I. 1992*, 2705–2707.
54. Belosludtsev, Y.Y., Kollah, R.O., Falck, J.R., and Capdevila, J.H. (1994) *Tetrahedron Lett. 35*, 5327–5330.
55. Demin, P.M., Vasiljeva, L.L., Belosludtsev, Y.Y., Myagkova, G.I., and Pivnitsky, K.K. (1990) *Bioorg. Khim. 16*, 571–572.
56. Lumin, S., Falck, J.R., Capdevila, J., and Karara, A. (1992) *Tetrahedron Lett. 33*, 2091–2094.
57. Demin, P.M., and Pace-Asciak, C.R. (1994) *207th ACS Meeting*, San Diego, March 13–17, Abstr #120, MEDI.

[Received October 28, 1994; accepted January 6, 1995]

Nuclear Magnetic Resonance Spectroscopic Analysis of Homoallylic and *bis* Homoallylic Substituted Methyl Fatty Ester Derivatives

Marcel S.F. Lie Ken Jie* and K.L. Cheng

Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong

ABSTRACT: Using a combination of selective irradiation ^{1}H nuclear magnetic resonance experiments and two-dimensional ^{1}H–^{13}C correlation spectroscopy spectral analysis of homoallylic and *bis* homoallylic substituted (azido, acetoxy, chloro and oxo) fatty ester derivatives, the carbon shifts of the ethylenic carbon atoms were determined. In the case of methyl 12-azido-9*Z*-octadecenoate (homoallylic), the carbon chemical shifts of the ethylenic C-9 and C-10 carbon nuclei are 133.092 and 124.596 ppm, respectively. In methyl 9-azido-12*Z*-octadecenoate (*bis* homoallylic), the carbon chemical shift of the ethylenic C-12 and C-13 carbon nuclei are 128.118 and 131.243 ppm, respectively.

Lipids 30, 115–120 (1995).

A survey of the chemical literature shows conflicting assignments of the ^{13}C carbon chemical shifts of the ethylenic carbon atoms of homoallylic substituted unsaturated fatty esters. Methyl ricinoleate (methyl 12-hydroxy-9*Z*-octadecenoate, **1a**) and *iso*-ricinoleate (methyl 9-hydroxy-12*Z*-octadecenoate, **1b**) are typical examples of oxygenated unsaturated fatty esters, which contain a homoallylic and a *bis* homoallylic hydroxy function in the alkyl chain, respectively. Wenkert *et al.* (1) have assigned the signal at 133.9 ppm to the shift of the C-9 ethylenic carbon nucleus, and the more upfield olefin signal at 125.8 ppm to the shift of the C-10 carbon atom of methyl ricinelaidate. Similar trends in the assignments of the shifts of such ethylenic carbon atoms with a hydroxy function at the homoallylic position have been reported by Bascetta and Gunstone (2), Dauzonne *et al.* (3), Radeglia *et al.* (4) and Bernassau and Fetizon (5).

Pfeffer *et al.* (6) have carried out an intensive study on the effects of homoallylic and *bis* homoallylic hydroxyl substitution on the olefinic ^{13}C resonance shifts in fatty esters. However, these investigators have used ^{13}C nuclear magnetic resonance (NMR) data from various literature sources, including those reported by Rakoff *et al.* (7), to make their assignments of olefinic resonances. Rakoff *et al.'s* (7) assignment of the carbon shifts of the C-9 and C-10 ethylenic carbon nuclei of methyl ricinoleate are in a reversed order, where the signals at 125.5 and 133.0 ppm have been assigned to the carbon shifts of the C-9 and C-10 ethylenic carbon atoms, respectively. This trend is supported by Carlson *et al.* (8) and Husain *et al.* (9). A further search of the chemical literature reveals that many authors concur with this reversed trend of assignment. For example, the shifts of C-3 and C-4 carbon atoms of 3*Z*-hexadecen-1-ol are assigned by Camps *et al.* (10) as 133.6 and 124.9 ppm, respectively. Barabas *et al.* (11) assign the shifts of C-3 and C-4 carbon atom of 3*Z*-hexen-1-ol as 135.3 and 125.3 ppm, respectively. The C-3 and C-4 carbon atoms in 3-octen-1-ol are also assigned in this order by Disselnkotter *et al.* (12). Gunstone (13) has acknowledged the ethylenic carbon shifts of methyl ricinoleate in this reversed order in his latest review.

We have recently addressed this anomaly by studying the two-dimensional ^{1}H–^{13}C correlation spectroscopy (COSY) NMR spectroscopic properties of the carbon chemical shifts of the ethylenic carbon atoms of methyl ricinoleate and methyl ricinelaidate (14). In this preliminary study, we have shown that the assignment of the shifts of the C-9 and C-10 ethylenic carbon atoms agree with those reported by Wenkert *et al.* (1). To substantiate our results, we consider it necessary to conduct additional NMR experiments on selected derivatives of methyl ricinoleate to confirm beyond all doubts the assignment of the ethylenic carbon atoms in molecules containing a homoallylic substituent. To achieve this goal, the hydroxy function of methyl ricinoleate is chemically transformed to an azido, acetoxy, chloro or oxo function. With this range of functional groups in the homoallylic position, it is possible to examine the different neighboring shift effects on the ethylenic carbon atoms and to observe the connectivities between the ^{1}H and ^{13}C shifts in the two-dimensional COSY spectra of these molecules. And, to extend our study to the *bis* homoallylic substituents on the carbon shifts of the ethylenic carbons, the azido, acetoxy, chloro and oxo derivatives of methyl *iso*-ricinoleate (methyl 9-hydroxy-12*Z*-octadecenoate) are also included in this investigation. Pfeffer *et al.*

*To whom correspondence should be addressed at the Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong.

Abbreviations: COSY, correlation spectroscopy; NMR, nuclear magnetic resonance; TMS, tetramethylsilane.

Copyright © 1995 by AOCS Press

(15) have recently published an amendment to their publication on the effects of *bis* homoallylic and homoallylic substitution on the olefin carbon resonance shifts in fatty acid methyl esters.

MATERIALS AND METHODS

The isolation of ricinoleic acid from castor oil and *iso*-ricinoleic acid from the seed oil of *Wrightia tinctoria*, and the procedures for the transformation of methyl ricinoleate (**1a**) and *iso*-ricinoleate (**1b**) to the corresponding azido (**2a, 2b**), acetoxy (**3a, 3b**), chloro (**4a, 4b**) and oxo (**5a, 5b**) derivatives are described elsewhere (16–19). NMR spectra were recorded on $CDCl_3$ solutions (0.2 mmol) with a JEOL (Tokyo, Japan) GSX-270 (270 MHz) spectrometer with proton observation at 270.00 MHz and carbon observation at 67.94 MHz. Chemical shifts are reported relative to tetramethylsilane (TMS) as internal standard. Connectivities in the ^{1}H–^{13}C NMR spectra were established at 270.05 MHz using a combination of spin–spin decoupling, COSY and double quantum filter experiments. Carbon atom types were established in ^{13}C NMR using a combination of broad-band proton decoupling and DEPT (distortionless enhancement by polarization transfer) experiments.

RESULTS AND DISCUSSION

The case of methyl 12-azido-9*Z*-octadecenoate (**2a**) is taken as an example to illustrate the detail NMR investigation leading to the confirmation of the assignment of the C-9 and C-10 ethylenic carbon atoms in this homoallylic substituted fatty ester derivative.

Focussing on the shifts of the protons of the α-methylene groups (8-*H* and 11-*H*) adjacent to the olefinic system, the ^{1}H NMR spectrum of methyl 12-azido-9*Z*-octadecenoate (Fig. 1a) shows the shift of the 8-*H* protons appearing at δ 2.04 as a multiplet. The shift of the 11-*H* protons also appears as a multiplet at δ 2.27. This slightly more downfield signal than that exhibited by the 8-*H* proton is due to the combined effects of the azide function and the olefinic bond. The methine proton (12-*H*) gives a quintet at δ 3.20. The olefinic protons (9-*H* and 10-*H*) appear as two multiplets centered at δ 5.40 and 5.53. To confirm the shifts of the olefinic protons, a selective double irradiation of the ^{1}H NMR spectrum was carried out at δ 2.2745 (the shift center of the 11-*H* proton), which caused the multiplet at δ 5.40 to collapse to a doublet (Fig. 1b). This result confirms the shift of the 10-*H* olefinic protons at δ 5.40. A similar selective irradiation experiment was carried at δ 2.0432 (the shift center of the 8-*H* proton), which caused the multiplet at δ 5.53 to collapse into a doublet (Fig. 1c). This observation confirms the multiplet at δ 5.53 as arising from the shift of the 9-*H* olefinic proton.

In the ^{13}C NMR spectrum of methyl 12-azido-9*Z*-octadecenoate, the carbon chemical shift of the C-8 and C-11 carbon nuclei appeared at 27.412 and 32.298 ppm, respectively. The shift of the olefinic carbon atoms were found at 124.596 and 133.092 ppm from TMS. From the two-dimensional ^{1}H–^{13}C COSY NMR spectrum (Fig. 2) the connectivities between the 8-*H*/C-8, 11-*H*/C-11 were readily established. With the proton shifts of the 9-*H* and 10-*H* protons already established through the selective irradiation experiments as described above, the carbon shift of the C-9 and C-10 carbon nuclei were therefore readily determined from the two-dimensional spectrum. The carbon shift of the C-9 carbon atom corresponded to the signal at 133.092 ppm, and that of the C-10 carbon atom to the signal at 124.596 ppm. The results of the ^{1}H and ^{13}C NMR of these derivatives are presented in Tables 1 and 2, respectively.

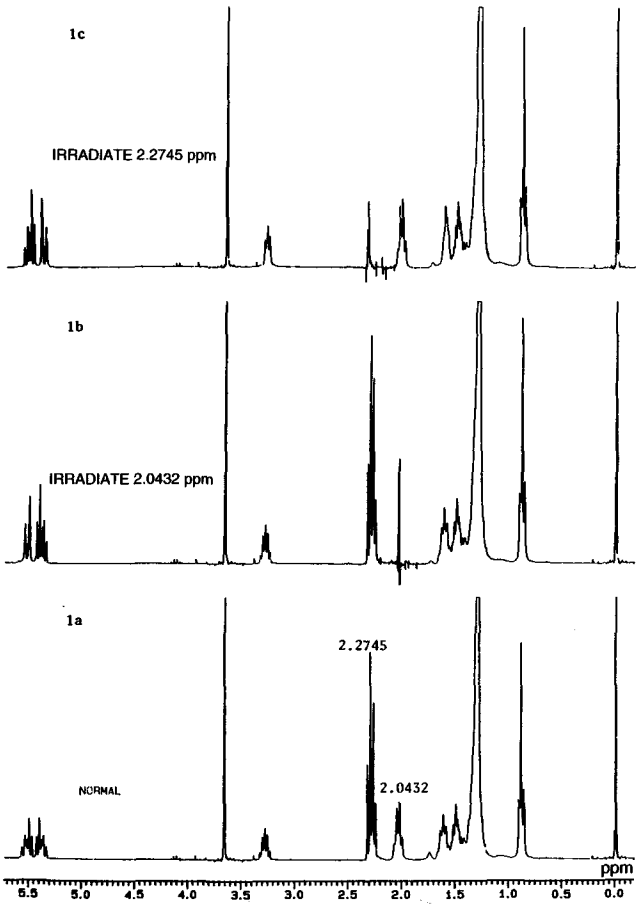


FIG. 1. ^{1}H nuclear magnetic resonance spectrum of methyl 12-azido-9*Z*-octadecenoate (**2a**).

Similar selective double irradiation experiments and two-dimensional ^{1}H–^{13}C COSY spectral analyses were carried out for the other derivatives [12-acetoxy (**3a**), 12-chloro (**4a**), 12-oxo (**5a**)] of methyl ricinoleate. In the ^{1}H NMR spectrum of methyl 12-acetoxy-9*Z*-octadecenoate, the resolution of the shifts of the olefinic protons was very distinct. In the case of the 12-chloro and 12-oxo derivatives, the signals (multiplets) for the olefinic protons were partially overlapped. Nevertheless, the selective irradiation experiments at the corresponding shift position of the 8-*H* and 11-*H* protons gave clear and distinct collapse of the associated signals in the olefinic re-

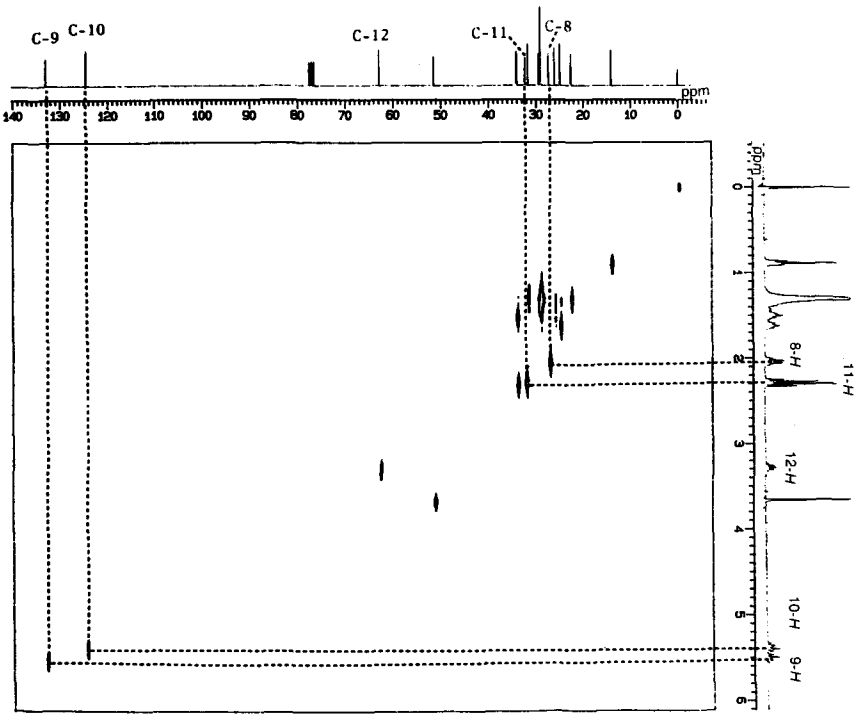
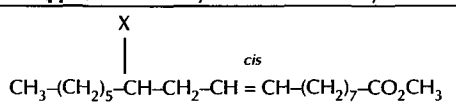


FIG. 2. ^{1}H–^{13}C correlation spectroscopy nuclear magnetic resonance spectrum of methyl 12-azido-9*Z*-octadecenoate (**2a**).

gion of the spectrum. This enabled the precise shift positions of the 9-*H* and 10-*H* olefinic protons in each case to be determined. By making use of the interconnectivities of the ^{1}H–^{13}C NMR COSY spectra of these derivatives, the carbon chemical shifts of the olefinic carbon atoms in each case were confirmed. The results of the ^{1}H and ^{13}C NMR spectral analysis of the acetoxy-, chloro- and oxo-derivatives of methyl ricinoleate are shown in Tables 1 and 2, respectively. The results of the ^{1}H and ^{13}C NMR spectroscopic analysis of methyl ricinoleate are also included in the tables for reference purpose. It can be concluded from this study that in the ^{1}H NMR spectroscopic analysis of homoallylic substituted unsaturated fatty ester derivatives, the effect of the substituent or function (such as hydroxy, azido, acetoxy, chloro and oxo) causes a more shielding effect on the γ-positioned than the δ-positioned olefinic proton. However, in the ^{13}C NMR analysis, the effect of the same substituents at the homoallylic position causes a significant shielding effect on the γ-olefinic carbon atom, but a deshielding effect on the more distally positioned δ-olefinic carbon atom.

In the *bis* homoallylic fatty ester derivatives of *iso*-ricinoleic acid, the substituent [9-azido (**2b**), 9-acetoxy (**3b**), 9-chloro (**4b**) and 9-oxo (**5b**)] is separated from the olefinic system by two methylene groups. The carbon chemical shifts of the α-methylene groups (C-11 and C-14) adjacent to the olefinic bond were readily distinguished and identified (Table 3). By making use of the two-dimensional ^{1}H–^{13}C COSY spectra of these derivatives, it was possible to establish the

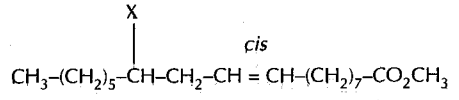
TABLE 1
^{1}H Nuclear Magnetic Resonance Chemical Shift Values (ppm) of Homoallylic Substituted Fatty Ester Derivatives (1a–5a)[a]



Compound	X	2-*H*	3-*H*	4-*H*/7-*H*	8-*H*	9-*H*	10-*H*	11-*H*	12-*H*	13-*H*	14-*H*/17-*H*	18-*H*	CO_2CH_3	
(**1a**)	OH	2.30(*t*)	b	a	2.05(*m*)	5.55(*m*)	5.40(*m*)	2.20(*m*)	3.60(*qn*)	b	a	0.88(*t*)	3.67(*s*)	1.70(*m*) OH
(**2a**)	N_3	2.30(*t*)	b	a	2.04(*m*)	5.53(*m*)	5.40(*m*)	2.27(*m*)	3.30(*qn*)	b	a	0.88(*t*)	3.67(*s*)	—
(**3a**)	OAc	2.30(*t*)	b	a	2.01(*m*)	5.45(*m*)	5.35(*m*)	2.30(*m*)	4.85(*qn*)	b	a	0.88(*t*)	3.67(*s*)	2.03(*s*) $OCOCH_3$
(**4a**)	Cl	2.30(*t*)	b	a	2.03(*m*)	5.50(*m*)	5.40(*m*)	2.48(*t*)	3.90(*m*)	b	a	0.89(*t*)	3.67(*s*)	—
(**5a**)	Oxo	2.30(*t*)	b	a	2.02(*q*)	5.57(*m*)	5.55(*m*)	3.15(*d*)	—	2.40(*t*)	a	0.88(*t*)	3.67(*s*)	—

[a]a, 1.20–1.50(*m*); b, 1.50–1.80(*m*); *m*, multiplet; *s*, singlet; *t*, triplet; *q*, quartet; *qn*, quintet.

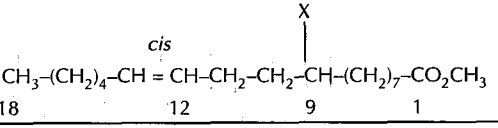
TABLE 2
^{13}C Nuclear Magnetic Resonance Chemical Shift Values (ppm) of Homoallylic Substituted Fatty Ester Derivatives (1a–5a)[a]



Compound	X	C-1	C-2	C-3	C-4/C-7	C-8	C-9	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C17	C-18	CO_2CH_3	$OCOCH_3$
(**1a**)	OH	174.343	34.093	24.937	a	27.389	133.217	125.280	35.377	71.514	36.874	25.743	a	31.866	22.641	14.100	51.463	—
(**2a**)	N_3	174.294	34.097	24.944	a	27.412	133.092	124.596	32.298	62.959	34.005	26.136	a	31.733	22.591	14.065	51.444	—
(**3a**)	OAc	174.294	34.097	24.952	a	27.320	133.629	124.267	31.962	74.020	33.627	25.376	a	31.759	22.599	14.073	51.444	170.821
(**4a**)	Cl	174.297	34.097	24.941	a	27.496	132.759	124.982	36.247	63.442	37.856	26.495	a	31.725	22.599	14.073	51.451	21.270
(**5a**)	Oxo	174.294	34.082	24.929	a	27.481	133.538	121.538	41.664	209.296	42.383	23.795	a	31.614	22.511	14.038	51.455	—

[a]28.829–29.463 ppm.

TABLE 3
^{1}H Nuclear Magnetic Resonance Chemical Shift Values (ppm) of *bis* Homoallylic Substituted Fatty Ester Derivatives (1b–5b)[a]



Compound	X	2-*H*	3-*H*	4-*H*/7-*H*	8-*H*	9-*H*	10-*H*	11-*H*	12-*H*	13-*H*	14-*H*	15-*H*/17-*H*	18-*H*	CO_2CH_3	
(**1b**)	OH	2.30(*t*)	a	b	a	3.61(*m*)	1.50(*m*)	2.15(*m*)	5.37(*m*)	5.38(*m*)	2.04(*m*)	b	0.89(*t*)	3.67(*s*)	1.80(*m*) O-*H*
(**2b**)	N_3	2.30(*t*)	a	b	a	3.24(*qn*)	1.53(*m*)	2.14(*m*)	5.33(*m*)	5.40(*m*)	2.04(*m*)	b	0.89(t)	3.67(*s*)	—
(**3b**)	OAc	2.30(*t*)	a	b	a	4.87(*qn*)	1.60(*m*)	2.04(*m*)	5.33(*m*)	5.36(*m*)	1.98(*m*)	b	0.89(*t*)	3.66(*t*)	2.04(s) $OCOCH_3$
(**4b**)	Cl	2.30(*t*)	a	b	a	3.85(*qn*)	1.73(*m*)	2.22(*m*)	5.31(*m*)	5.42(*m*)	2.05(*m*)	b	0.89(*t*)	3.66(*s*)	—
(**5b**)	Oxo	2.30(*t*)	a	b	2.40(*t*)	—	2.40(*t*)	2.30(*m*)	5.31(*m*)	5.37(*m*)	2.03(*m*)	b	0.89(*t*)	3.66(*s*)	—

[a]a, 1.50–1.80(*m*); b, 1.80–1.50(*m*); *m*, multiplet; *s*, singlet; *t*, triplet; *qn*, quintet.

precise proton chemical shifts of the 11-*H* and 14-*H* protons. The sample was subjected to selective irradiation at the corresponding shift positions of 11-*H* and 14-*H*. From these experiments, the proton chemical shifts of the olefinic protons in the *bis* homoallylic system were determined by observing the collapse or simplification of the olefinic proton signals. As an example, the shifts of the 11-*H* and 14-*H* of methyl 9-azido-12*Z*-octadecenoate (**2b**) appeared at δ 2.144 and 2.041, respectively. Selective irradiation at δ 2.144 caused the olefinic signal at δ 5.334 to collapse, which determined the shift of the 12-*H* olefinic proton. Likewise, by irradiating at δ 2.041, the shift of the 13-*H* olefinic proton was found at δ 5.399. Referring to the two-dimensional COSY spectrum, the interconnectivities allowed the carbon shifts of C-12 and C-13 to be established at 128.118 and 131.243 ppm. From this study, it can be concluded that a *bis* homoallylic substituent (hydroxy, azido, acetoxy, chloro or oxo) causes a greater shielding effect on the δ-positioned olefinic carbon atom than on the more distally ε-positioned olefinic carbon atom. The results of the ^{1}H and ^{13}C NMR analysis of the various derivatives of methyl *iso*-ricinoleate are presented in Tables 3 and 4, respectively. The assignment of the shifts of the olefinic carbons of methyl *iso*-ricinoleate agrees with those reported by Pfeffer *et al.* (6).

It can be concluded that for a $C_n=C_{n+1}$ bond, the carbon chemical shift of the C_n is always greater than that of the C_{n+1} in a homoallylic [$C_n=C_{n+1}$-CH_2-CH(X)-, where X = OH, N_3, OAc, Cl or oxo] or *bis* homoallylic [$C_n=C_{n+1}$-CH_2-CH_2-CH(X)-] system.

ACKNOWLEDGMENTS

The Lipid Research Fund and the Committee on Research Grants of the University of Hong Kong provided financial assistance.

REFERENCES

1. Wenkert, E., Buckwalter, B.L., Burfitt, I.R., Gasic, M.J., Gottlieb, H.E., Hagaman, E.W., Schell, F.M., and Wovkulich, P.M. (1976) in *Topics in Carbon-13 NMR Spectroscopy,* vol. 2 (Levy, G.C., ed.) John Wiley & Sons, New York.
2. Bascetta, E., and Gunstone, F.D. (1985) *Chem. Phys. Lipids 36,* 253–261.
3. Dauzonne, D., Demerseman, P., Egyed, J., Lang, C., Platzer, N., and Royer, R. (1977) *Tetrahedron 33,* 19–25.
4. Radeglia, R., Poleschner, H., and Heydenreich, M. (1991) *Magnetic Res. Chem. 29,* 1028–1035.
5. Bernassau, J.M., and Fetizon, M. (1981) *Tetrahedron 37,* 2105–2109.
6. Pfeffer, P.E., Sonnet, P.E., Schwartz, D.P., Osman, S.F., and Weisleder, D. (1992) *Lipids 27,* 285–288.
7. Rakoff, H., Weisleder, D., and Emken, E.A. (1979) *Lipids 14,* 81–83.
8. Carlson, K.D., Chaudhry, A., Peterson, R.E., and Bagby, M.O. (1990) *J. Am. Oil Chem. Soc. 67,* 495–498.
9. Husain, S., Kifayatullah, M., Sastry, G.S.R., and Raju, N.P. (1993) *J. Am. Oil Soc. 70,* 1251–1254.
10. Camps, F., Hospital, S., Rosell, G., Delgado, A., and Guerrero, A. (1992) *Chem. Phys. Lipids 61,* 157–167.
11. Barabas, A., Botar, A.A., Gocan, A., Popovici, N., and Hodosan, F. (1978) *Tetrahedron 34,* 2191–2194.

TABLE 4
^{13}C Nuclear Magnetic Resonance Chemical Shift Values (ppm) of *bis* Homoallylic Substituted Fatty Ester Derivatives (1b–5b)[a]

CH_3-$(CH_2)_4$-CH = CH-CH_2-CH(X)-CH_2-$(CH_2)_7$-CO_2CH_3 (*cis*)

18 12 9 1

Compound	X	C-1	C-2	C-3	C-4/C-6	C-7	C-8	C-9	C-10	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	CO_2CH_3	$OCOCH_3$
(**1b**)	OH	174.355	34.089	24.918	a	25.571	37.325	71.678	37.432	23.604	129.184	130.654	27.210	a	31.538	22.588	14.080	51.466	—
(2b)	N3	174.259	34.067	24.910	a	26.056	34.330	62.495	34.426	23.867	128.118	131.243	27.206	a	31.534	22.591	14.080	51.455	—
(**3b**)	OAc	174.267	34.086	24.937	a	25.242	34.097	73.985	34.151	23.191	128.576	130.693	27.183	a	31.557	22.595	14.084	51.432	170.848
																			21.264
(**4b**)	Cl	174.282	34.074	24.910	a	26.427	38.452	63.564	38.517	24.284	127.980	131.388	27.232	a	31.530	22.584	14.084	51.459	—
(**5b**)	Oxo	174.259	34.055	24.879	a	23.714	42.681	210.801	42.860	21.728	127.778	131.231	27.160	a	31.519	22.580	14.077	51.459	—

[a]28.829–29.463 ppm.

12. Disselnkotter, H., Eiter, K., Karl, W., and Wendisch, D. (1976) *Tetrahedron 32*, 1591–1595.
13. Gunstone, F.D., in *Advances in Lipid Methodology—Two* (1993) p. 13 (W.W. Christie, ed.) The Oily Press, Dundee.
14. Lie Ken Jie, M.S.F., and Cheng, K.L. (1993) *Nat. Prod. Letters 3*, 65–69.
15. Pfeffer, P.E., Sonnet, P.E., Schwartz, D.P., Osman, S.F., and Weisleder, D. (1994) *Lipids 29*, 913.
16. Lie Ken Jie, M.S.F., and Lam, C.H. (1977) *Chem. Phys. Lipids 20*, 1–12.
17. Lie Ken Jie, M.S.F., Chan, H.W.M., Wai, J.S.M., and Sinha, S. (1981) *J. Am. Oil Chem. Soc. 58*, 705–706.
18. Lie Ken Jie, M.S.F., and Ahmad, F. (1983) *J. Am. Oil Chem. Soc. 60*, 1783–1785.
19. Lie Ken Jie, M.S.F., and Lao, H.B. (1987) *Chem. Phys. Lipids 45*, 65–74.

[Received October 26, 1994; accepted January 16, 1995]

Phase Properties of Mixtures of Ceramides

Chien-Hsuan Han[a], Ronald Sanftleben[b] and Timothy S. Wiedmann[a,*]

[a]Department of Pharmaceutics, College of Pharmacy, University of Minnesota, and [b]CIMA Laboratories, Minneapolis, Minnesota 55455

ABSTRACT: Ceramides have been proposed to have a central role in the function of the stratum corneum. Ceramides also influence the phase properties of model skin lipid mixtures, but the relevance of this to the stratum corneum function is controversial. Because the stratum corneum contains several classes of ceramides, the type of ceramides used in model mixtures of stratum corneum lipid lamellae may be important. Thus, the properties of α-hydroxy fatty acid containing (HFAC) and nonhydroxy fatty acid containing (NFAC) ceramides and their mixtures have been investigated. Ceramides were obtained by the conversion of purified bovine brain cerebrosides. Isolated, anhydrous HFAC underwent an endothermic solid to liquid transition at 92°C. With hydration, an endothermic transition at 71.8°C was observed which was accompanied by a reduction in the birefringence. The enthalpy increased from 66 to 89 J/g with a 20-d storage time. These thermal properties are very similar to those observed with hydroxy fatty acid containing cerebrosides. In contrast, anhydrous nonhydroxy fatty acid containing ceramides underwent a broad endothermic transition over the temperature range of 50–90°C. When hydrated, the initial endothermic transition was interrupted by an exothermic transition that was followed immediately by a second endothermic transition. During these thermal changes, there was a loss of birefringence, and with completion of the second endothermic transition, a nonbirefringent liquid was observed. NFAC samples, stored at 70°C for 5 min, cooled, and then rescanned, displayed only one endotherm at 75°C. The thermal behavior of mixtures of HFAC and NFAC was relatively simple, with a progressive decrease and broadening in the temperature of the phase transition as the proportion of NFAC increased up to weight fractions of NFAC of 0.7. At lower weight fractions, a plateau in the transition temperature as a function of weight fraction was observed. Even at a weight fraction of 0.1 HFAC, no transition to a nonbirefringent liquid state was observed. The high enthalpic changes observed with mixtures of HFAC and NFAC are consistent with the proposed central role that ceramides have in the mechanical and permeability properties of the skin. Moreover, the marked difference in the properties of these two similar lipids may help to explain some of the properties of the stratum corneum.

Lipids 30, 121–128 (1995).

*To whom correspondence should be addressed at University of Minnesota, College of Pharmacy, 308 Harvard St. SE, Minneapolis, MN 55455.

Abbreviations: FID, flame-ionization detector; HFAC, hydroxy fatty acid containing ceramides; HFA–CER, hydroxy fatty acid containing cerebrosides; NFAC, nonhydroxy fatty acid containing ceramides; NFA–CER, nonhydroxy fatty acid containing cerebrosides; TLC, thin-layer chromatography.

Ceramides are N-acyl linked sphingolipids which are found only in abundance in the stratum corneum (1). Following isolation from the skin, the ceramides have been separated by thin-layer chromatography (TLC) into five to seven classes based on the number of hydroxyl moieties and acyl chain-length (2–4). Aside from the unusual acyloxy derivative which is present at a level of 10%, the remaining ceramides are characterized by a relatively small polar head group of two to four hydroxyl moieties and an amide linkage connecting the fatty acid to the sphingolipid base (4). The common acyl chains are 16 and 24 to 28 carbons in length.

Ceramides have been suggested to have a central role in the function in the skin by contributing to the water impermeability of the stratum corneum (5–8). In addition, ceramides have been implicated in controlling epidermal turnover (5). The multiple hydroxyl moieties could form an extensive hydrogen-bonded network which contributes to the interbilayer attractive interactions (9–12). The asymmetric chainlengths coupled with the terminal hydroxyl groups may also form an interdigitated multilamellar arrangement and thereby provide cohesive links in the intercellular region of the stratum corneum (8,13,14).

While ceramides are evidently important to the skin, the details of their role in the phase behavior and how this relates to the function of the stratum corneum remain unresolved (15–18). Part of the difficulty may lie in the use of different model lipid mixtures as well as various sources of ceramides. In particular, type III ceramides derived from bovine brain sphingomyelin have been commonly used as the sole ceramide which only has two hydroxyl moieties. It would seem that a better understanding of the properties of ceramides would prove useful as the first step in resolving the controversy regarding the role of ceramides in the stratum corneum. Therefore, in this study, the thermotropic phase behavior of nonhydroxy fatty acid containing (NFAC) ceramides and hydroxy fatty acid containing ceramides (HFAC) has been determined. In addition, the phase properties of the ceramide mixtures have been investigated to obtain the fundamental basis of the role of ceramides in the lipid lamellae of the stratum corneum.

MATERIALS AND METHODS

Isolation of ceramides. Cerebrosides were isolated from bovine brain following a modified procedure of Radin (19).

Copyright © 1995 by AOCS Press

Briefly, brains were dehydrated by blending twice with acetone (4 mL/g). The dispersion was allowed to settle and then was filtered through Whatman filter paper in a Buchner funnel. The cakes were further dried overnight in a dessicator attached to the house vacuum system. The dried mass was dispersed in 85% ethanol in water by blending and then refluxed for 30 min. The solids were collected by filtration and refluxed again with 85% ethanol. The combined supernatants were placed in a refrigerator. The resulting precipitate was collected by filtration, dissolved in a minimum volume of warm ether, and precipitated with acetone (6:1 volume ratio to ether). After storing overnight in the refrigerator, a Folch extraction ($CHCl_3/CH_3OH/H_2O$, 4:2:1) was introduced. This minimized the clogging of the subsequent dichloromethane/methanol solvent gradient column (Bio-Sil A, 100–200 mesh; BIO-RAD, Richmond, CA) that was used to purify the cerebrosides. The cerebrosides eluted at 10% methanol in dichloromethane.

The isolated cerebrosides were then converted to ceramides by cleaving the carbohydrate moiety from the sphingosine base by the procedure of Carter *et al.* (20). The resulting ceramides were separated into two groups according to the absence or presence of hydroxy fatty acids by silica column chromatography in a dichloromethane/methanol solvent gradient. The NFAC fraction began to elute at 4% methanol with the HFAC fraction following.

The acyl chain composition of NFAC was determined by reacting the dried fraction with 10% BCl_3 in methanol for 20 min at 80°C. The resulting fatty acid methyl esters were extracted into hexane, dried under argon, and reconstituted with carbon disulfide. The solution was injected on a gas chromatograph (model 5830A; Hewlett-Packard, Avondale, PA) equipped with a flame-ionization detector (FID) and 18850A GC terminal for automatic peak integration. The solid phase consisted of 10% DEGS-PS on 80/100 Supelcoport (Supelco, Inc., Bellefonte, PA) packed in a ⅛″ × 30 cm column. The carrier gas was N_2 at a flow rate of 20 mL/min and the injector, column and detector temperatures were 250, 200 and 275°C, respectively. The composition was determined by comparing the retention times with known standards, and the integrated areas were corrected for the differences in response of the FID. Other peaks were assigned by interpolation of plots of the log of the retention time as a function of carbon number.

Optical microscopy/birefringence. Visual changes occurring in the samples with fixed and programmed temperature variations were viewed with a hot stage microscope. A WILD (Heerbrugg, Switzerland) M3Z stereomicroscope, MPW51 shutter piece, and Photoautomat MPS45 using center-weighted integrated metering were coupled to a Mettler (Highstown, NJ) FP800 Thermosystem. Also included was a Mettler FP80 central processor for programmed temperature control and a Mettler FP82HT hot stage. Birefringence was viewed by placing Tiffen 49 mm SR polarizing lenses (Hauppauge, NY) above and below the sample. The angle between the direction of the polarizers was visually set at right angles by minimizing the transmitted light. Photographs were taken with Kodak ASA 400 black and white 35 mm film (Rochester, NY). Typical exposure times were 30 to 60 s for the low birefringent samples.

Thermal analysis. Ceramides were first lyophilized from cyclohexane to obtain a dry powder. Accurately determined weights of HFAC and NFAC were combined to generate the entire range of compositions. The mixtures were dissolved in 10–30% methanol in dichloromethane and the appropriate volume was placed into preweighed coated aluminum differential scanning calorimeter pan (DuPont, Wilmington, DE). The solution was dried overnight under high vacuum and excess distilled water was added to bring the solid content to less than 33 wt%. The pans were then hermetically sealed. Uniform hydration was achieved by heating in a steam bath (85°C) for 20 min. Due to the presence of metastability, variable incubation conditions were used. TLC failed to indicate any chemical stability problems with these procedures (21).

Calorimetric scans were usually performed at 5°/min on a DuPont 910 differential scanning calorimeter (Wilmington, DE). The phase transition was determined by extrapolation to the baseline of the most rapid rise in the excess heat capacity as a function of temperature curve. This value is more reproducible than the maximum value of the excess heat capacity as a function of temperature curve and compensates for the rapid scan rate. The enthalpy was determined by integrating the area under the curve from a plot of the excess heat capacity as a function of temperature. For the phase diagram, onset and completion temperatures were corrected by the method of Mabrey and Sturtevant (22) using the endotherm obtained with pure HFAC.

RESULTS

Both fractions of the isolated ceramides yielded a single spot with TLC in a solvent system of 9:1 chloroform/methanol. The fatty acid composition of the NFAC are given in Table 1. Also included are the results from nonhydroxy fatty acid containing and hydroxy fatty acid containing cerebrosides (NFA–CER and HFA–CER, respectively) as reported by O'Brien and Rouser (23). The major fatty acids are stearic, lignoceric and nervonic, which make up about 60% of the fatty acids on NFAC.

In Figure 1, the differential scanning calorimetric results of anhydrous and hydrated samples of HFAC are given. HFAC underwent a solid to liquid phase change at 92°C. The transition was independent of whether the solid was a lyophilized powder or cooled from a liquid state. With hydration, the transition temperature was 71.8 ± 0.4°C, which is in good agreement with that reported for HFAC obtained with the unmodified Radin procedure (21). Because the enthalpy increased with storage time, further study was needed as described in the following.

HFAC were viewed with optical microscopy while heating. HFAC were nearly transparent both above and below the phase transition temperature. The birefringence was also determined, and the results are given in Figure 2. At 40°C, HFAC was found

TABLE 1
Acyl Chain Composition of Nonhydroxy Fatty Acid Containing Cerebrosides, Nonhydroxy Fatty Acid Containing Ceramides and Hydroxy Fatty Acid Containing Cerebrosides

Acyl	NFA–CER[a]	NFAC[b]	HFA–CER[c]
12:0	—	—	0.4
13:0	—	—	0.2
14:0	0.2	—	0.8
15:0	trace	—	trace
16:0	3.5	3.4	0.1
16:1	0.1	—	tr
17:0	trace	—	0.2
18:0	5.8	11.2	16.2
18:1	2.7	—	—
19:0	0.2	0.5	0.4
20:0	0.6	1.1	0.5
20:1	0.3	—	—
22:0	3.6	5.4	5.8
22:1	0.6	—	—
23:0	5.2	5.8	8.9
23:1	1.2	1.0	—
24:0	17.4	14.1	30.1
24:1	39.3	37.1	14.6
25:0	3.6	3.0	7.0
25:1	6.2	4.8	3.0
26:0	1.7	0.6	3.9
26:1	7.6	4.0	6.3

[a]Nonhydroxy fatty acid–containing cerebrosides from bovine brain reported by O'Brien and Rouser (23).
[b]Nonhydroxy fatty acid–containing ceramides, this study.
[c]Hydroxy fatty acid–containing cerebrosides from bovine brain reported by O'Brien and Rouser (23).

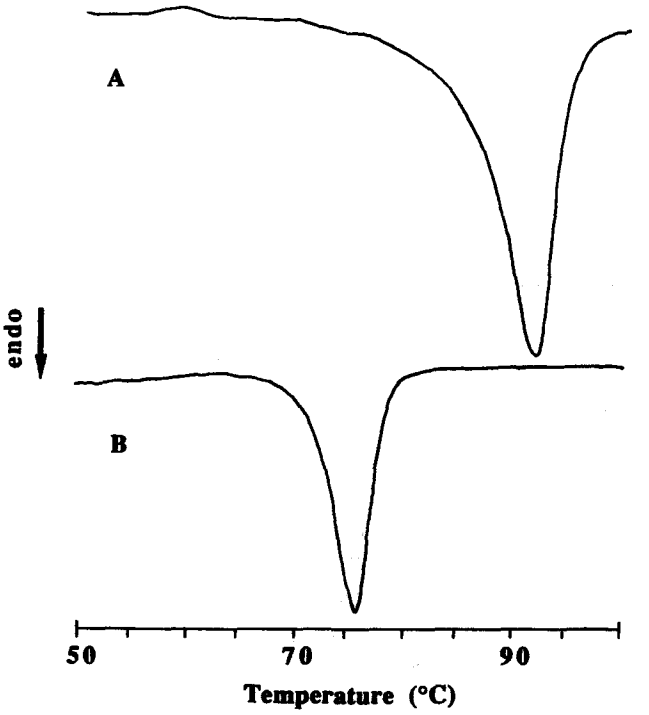


FIG. 1. Calorimetric scans at 5°C/min of (A) anhydrous hydroxy fatty acid ceramide, (B) hydrated hydroxy fatty acid ceramide (66 aqueous wt%).

to be brightly birefringent (Fig. 2A). With heating, there was a reduction in the birefringence over the temperature range of 65–75°C. After completion of the thermal changes, the sample still remained somewhat birefringent (Fig. 2B). As can be noted, considerable heterogeneity with regard to the intensity of the birefringence is evident for HFAC.

When the storage time of HFAC was increased, neither the overall shape of the curve nor the position of the peak temperatures changed. However, the integrated area under the curve progressively increased over a 20-d period as is shown in Figure 3. Immediately rescanning the sample after rapid cooling from 90°C resulted in an enthalpy change of about 66 J/g. After 20 d of storage, the enthalpy change for the thermal transition appeared to reach a plateau value of 89 ± 11 J/g.

A similar series of experiments was also conducted with the NFAC. The solid-to-liquid phase change of a lyophilized powder of NFAC occurred over a broad temperature range (Fig. 4A). With hydration, relatively complicated thermal changes were observed (Fig. 4B). At 55°C, NFAC began to undergo an endothermic transition. This was interrupted by an exothermic transition that had a peak occurring at 72°C. Thereafter, a second endothermic transition was recorded which had a peak temperature of about 79°C. Other samples prepared with NFAC displayed similar behavior although the peak temperatures were variable.

Hydrated NFAC was also nearly transparent at 40°C. As the temperature was increased, melting to a transparent liquid

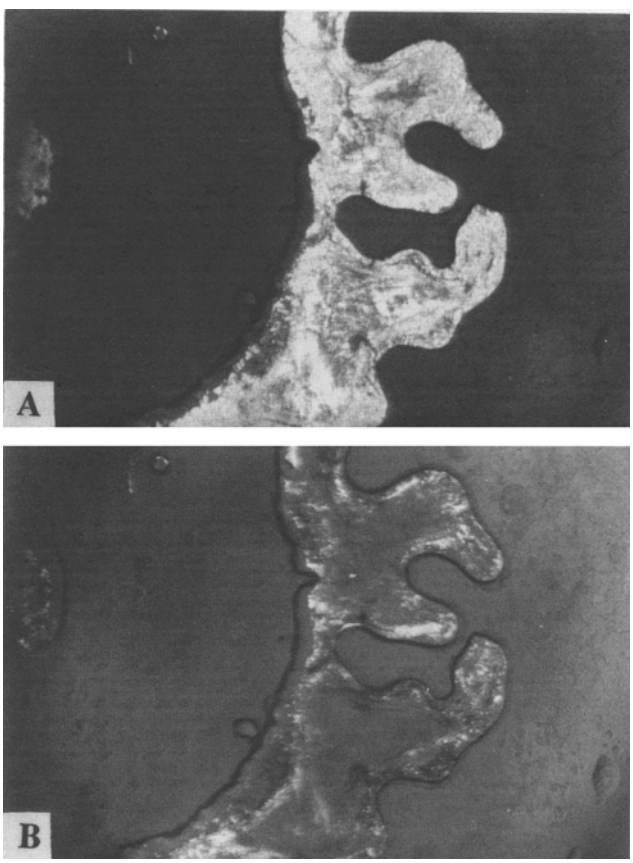


FIG. 2. Photographs of hydrated hydroxy fatty acid ceramide taken with cross-polarizers at temperatures of (A) 40°C and (B) 79°C.

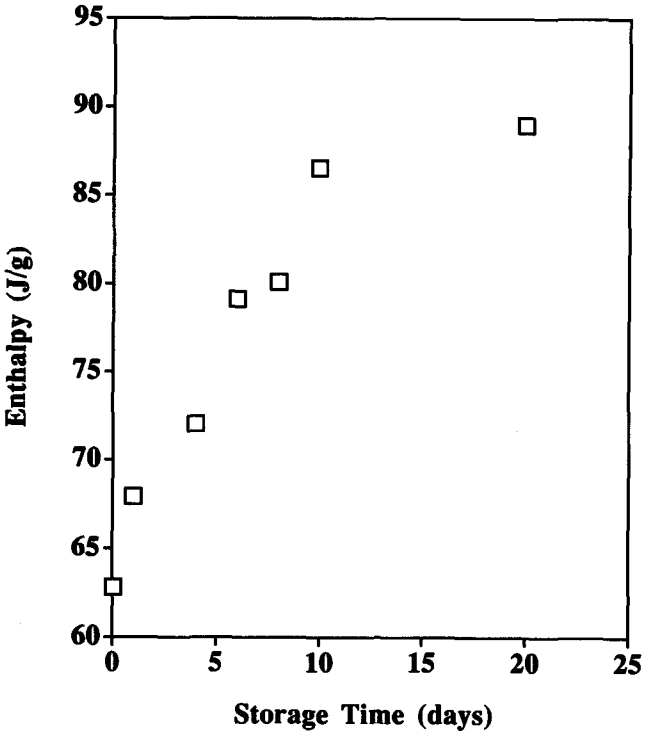


FIG. 3. The change in enthalpy of hydroxy fatty acid ceramides determined as a function of room temperature storage time.

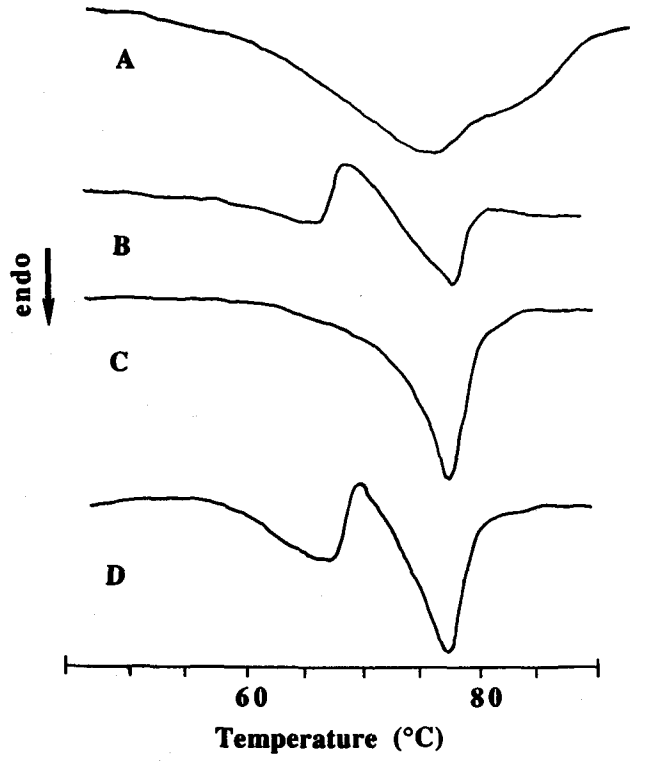


FIG. 4. Calorimetric scans at 5°C/min of (A) anhydrous nonhydroxy fatty acid ceramide and (B) hydrated nonhydroxy fatty acid ceramide (66 aqueous wt%). Calorimetric scans of hydrated nonhydroxy fatty acid ceramide obtained with the following sequential conditions: (C) heated to 70°C, cooled to 40°C and then scanned at 5°C/min and (D) cooled from 90°C and then immediately scanned at 5°C/min.

occurred over the temperature range of 75–80°C. In addition, NFAC was brightly birefringent at 40°C as is evident in Figure 5A. The birefringence remained intense until a temperature of 70°C (Fig. 5B), after which there was a loss in intensity. This corresponded to the formation of a nonbirefringent liquid (Fig. 5C). The birefringence was partially recovered with cooling to 40°C, although reappearance of the bright birefringence took a longer time.

The thermograms observed for NFAC are indicative of metastability, and thus the effect of sample history on the thermal response was determined. NFAC was scanned at 5°C/min

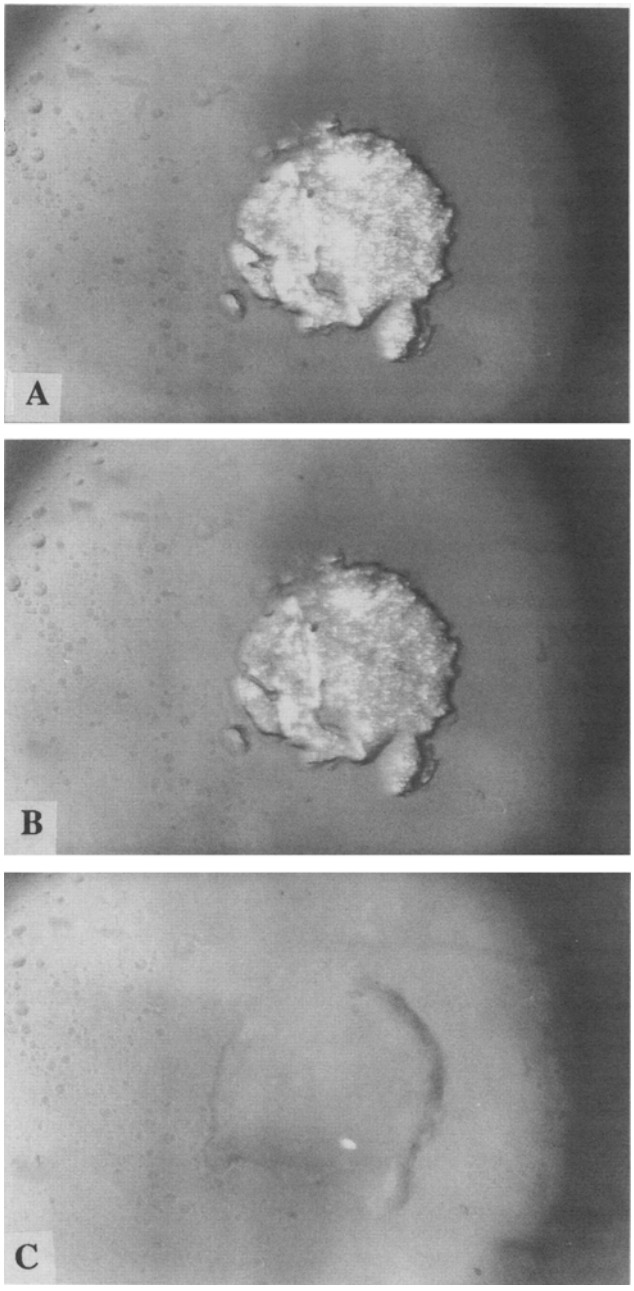


FIG. 5. Photographs of hydrated nonhydroxy fatty acid ceramide taken with cross-polarizers at temperatures of (A) 40°C, (B) 70°C and (C) 85°C.

to a temperature of 90°C which yielded the result shown in Figure 4B. After cooling to room temperature, the sample was heated at 5°/min to 70°C, held at this temperature for 5 min, and then cooled to room temperature. The sample was then scanned at 5°C/min to a final temperature of 95°C with the resulting thermogram given in Figure 4C. Only one endothermic transition was observed which had a temperature of 75°C. Finally, the sample was cooled to 40°C and scanned to 90°C, and the metastability was again observed (Fig. 4D).

Finally, thermal analysis of HFAC and NFAC mixtures was conducted. In Figure 6, the endothermic transitions are given for samples that were stored for 7 d. The top scan shows the trace of pure HFAC which produced a single endotherm. Incorporation of NFAC into HFAC caused a broadening of the curves and a slight reduction in the onset and completion temperatures. At small weight fractions of HFAC, the value of the peak temperature of the transition reached a plateau. The bottom trace was obtained with pure NFAC. This was the only sample where an exothermic peak was observed and which formed a liquid. Samples containing 0.7 and 0.22 weight fraction HFAC had time-dependent enthalpy changes and time-independent transition temperatures comparable to that observed with pure HFAC.

In Figure 7, the corrected onset and completion temperatures are given as a function of the weight fraction of HFAC. The progressive decrease in onset and completion temperature with increasing amounts of NFAC is clearly seen. At low HFAC weight fractions, there is a plateau in the onset and completion temperatures. Overall, HFAC and NFAC evidently do not exhibit complex phase behavior over most of the composition range.

DISCUSSION

This study represents the first investigation into the thermal behavior of mixtures of HFAC and NFAC. Each class still contains many different chemical species, due to the variability in the fatty acid chain with respect to the length and degree of unsaturation. Nevertheless, rather simple thermal behavior is observed. Thus, in this respect it shares commonality with lipid mixtures, such as egg phosphatidylcholines and bovine brain cerebrosides.

The modified procedure for isolating the ceramides is straightforward. The use of the acetone dehydration step followed by the ethanol extraction is much easier than the chloroform/methanol extractions suggested by Carter *et al.* (20). Moreover, the Folch extraction introduced before the column purification of the cerebrosides apparently removed polar impurities that contributed to the precipitation on the column. The fatty acid composition of the nonhydroxy containing ceramides is in very good agreement with that reported for the nonhydroxy containing cerebrosides. This suggests the minor modification does not influence the resulting composition of ceramides. Moreover, the fatty acid composition of the HFAC is expected to closely follow that of the HFA–CER also given in Table 1.

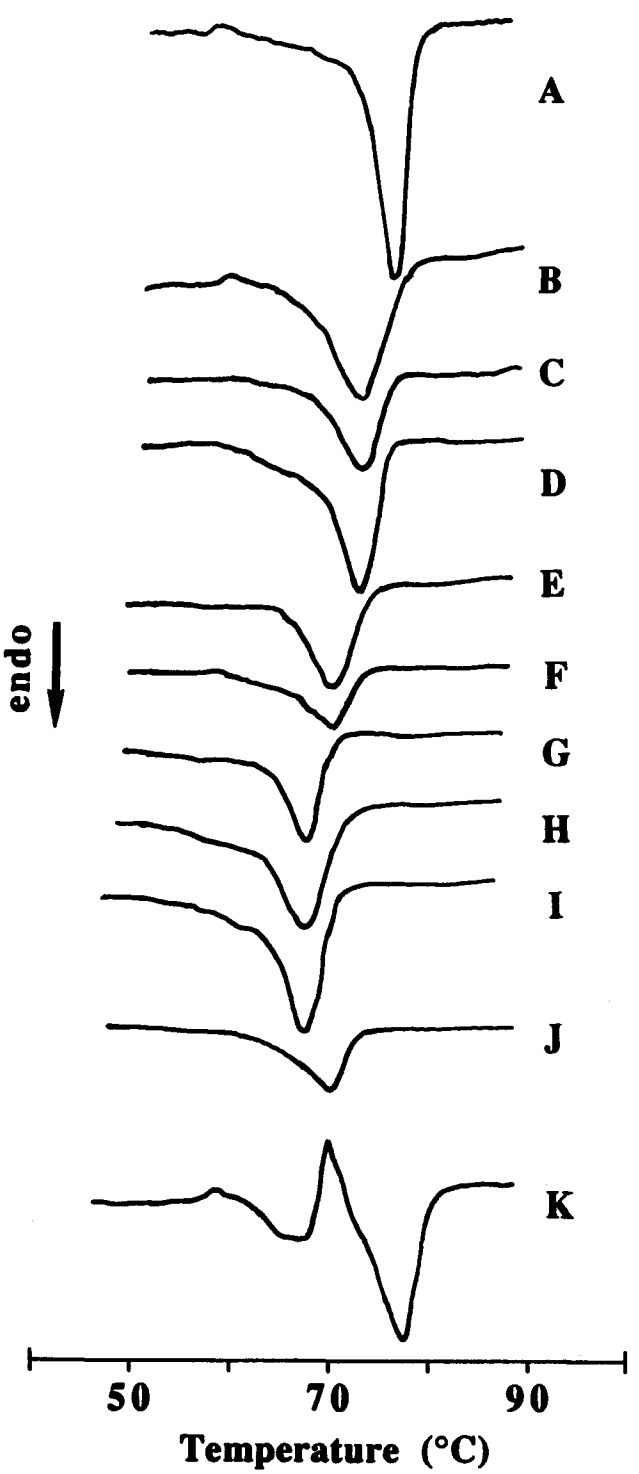


FIG. 6. Calorimetric scans at 5°C/min of mixtures of hydroxy fatty acid ceramides and nonhydroxy fatty acid ceramides stored for 7 d at room temperature with weight fractions of hydroxy fatty acid ceramides of (A) 1.0, (B) 0.90, (C) 0.80, (D) 0.74, (E) 0.60, (F) 0.50, (G) 0.39, (H) 0.29, (I) 0.21, (J) 0.10 and (K) 0.0.

The thermal behavior of the anhydrous and hydrated samples coupled with the measurement of the birefringence suggests that hydrated HFAC samples undergo a gel-to-liquid crystalline phase transition. The temperature and enthalpy of

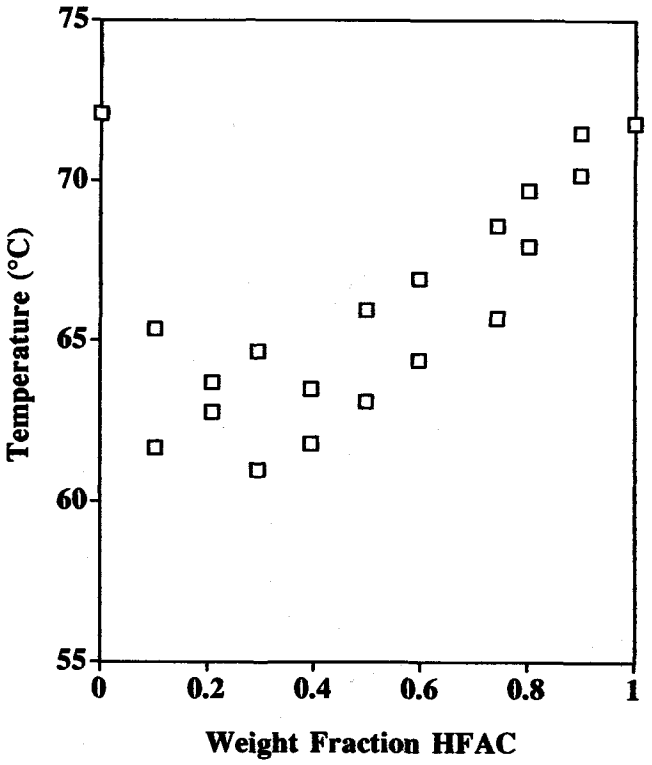


FIG. 7. Corrected onset and completion temperatures given as a function of weight fraction of hydroxy fatty acid ceramides. HFAC, hydroxy fatty acid containing ceramides.

the thermal transition of HFAC are in reasonable agreement with Shah *et al.* (24) who reported values of 80.0°C and 8.5 kcal/mol. However, they indicated that there was a gel to inverted hexagonal phase transition. Until more details become available, it is not possible to determine the source of this discrepancy.

While the temperature of the transition of HFAC remained constant, the enthalpy change continued to increase for at least 20 d. Analogous results have been reported for bovine brain HFA-CER (25,26). However, the enthalpy for the gel to liquid crystalline phase transition was still increasing after six months (26).

Curatolo and Jungalwala (26) suggested that the time-dependent enthalpy change of HFA-CER was the result of the slow formation of a stable gel state from a metastable state. They further speculated that the difference in these two states lay in the extent of order of the acyl chain. That is, the presence of the α-hydroxyl moiety on the acyl chain retarded the rate of formation of a highly ordered chain. A similar phenomenon may be occurring with the HFAC.

Alternatively, there may be a slow dehydration occurring in the interbilayer aqueous space as has been suggested for phospholipids (26,27). This would in turn have significance for the role of ceramides in the function of the lipid lamellae of the stratum corneum. X-ray diffraction studies of intact stratum corneum have revealed that the lipid lamellar spacing does not appreciably change with increasing water content (28). This suggests that the lipid lamellae have sufficiently strong attractive interactions to prevent the swelling which may in part be due to the presence of ceramides.

The magnitude of the enthalpy change is also noteworthy. Using the reported acyl chain composition to calculate the average molecular weight, the enthalpy change after storage is about 15 kcal/mol (25,26). Therefore, the enthalpy change of the transition is almost twice as high as that observed for phosphatidylcholines which typically is 8 kcal/mol. For HFA-CER, Curotolo and Jungalwala (26) observed an enthalpy change of 66.5 J/g after 3 months of storage. This is about two-thirds of the value measured for HFAC. Thus, there is a significant energetic contribution from disruption of the intermolecular hydrogen bonding between the lipid head groups with generation of liquid crystalline state.

Overall, the time independence of the transition temperature and the magnitude and time dependence of the enthalpy change of HFAC are similar to cerebrosides. This similarity has the implication that the functional groups common to both lipids dominate the phase behavior. Therefore, it may be that the neutral carbohydrate moiety of cerebrosides does not significantly change the nature of the phases above and below the transition temperature. This argument is corroborated by Curotalo's observations that the phase transition temperatures and enthalpies were largely independent of fatty acid chain-length (26).

For NFAC, evidently a metastable gel phase is readily produced. No estimate of the enthalpy change was obtained due to the proximity of the exothermic transitions. Shah *et al.* (24) reported similar complicated thermal behavior of their NFAC. From X-ray diffraction, it appears that NFAC exists as a lamellar crystalline structure at low temperatures, but above the thermal transitions only reflections at 30 and 4.6 Å remain. An earlier report of the X-ray crystallography performed with N-palmitoyl-galactosyl-sphingosine may also be relevant (29). With this lipid, a low-temperature metastable phase was produced by cooling from the liquid crystalline phase. The actual metastability was postulated to arise from the presence of water about the head groups of the lipids. The stable state was characterized as a dehydrated lamellar phase. Bovine brain NFA–CER also displayed analogous metastable behavior (25,26,30–32). Thus, there may be a connection between the metastable behavior of NFAC and the extent of hydration.

It is of particular interest that HFAC undergoes a transformation to an ordered phase while NFAC does not. NFAC differs from HFAC by a single hydroxyl group in the head group. As has been established for some time, the stereochemistry (D-isomer) of the naturally occurring α-hydroxy moiety on fatty acids is such that only intermolecular hydrogen bonding is allowed (10). The intermolecular hydrogen bonding could provide a stabilizing effect for the lamellar phase. Furthermore, the additional hydroxyl group may also interact with water to maintain a hydrated head group. The cerebrosides contain an additional carbohydrate moiety in comparison to NFAC and undergo a gel-to-liquid crystalline phase transition. The presence of only an amide and two hy-

droxy moieties on NFAC may be insufficient to maintain the hydration of the head group, and thus there is a loss of the lamellar structure at high temperatures.

In spite of the complexity of the thermal response of HFAC and NFAC, mixtures of NFAC and HFAC yield relatively simple thermograms. Here again, the behavior of ceramides appears to mirror that observed with mixtures of cerebrosides (25,26). While progressively more complicated thermograms were obtained with fractionation and purification of individual cerebrosides, the crude mixtures yielded single endothermic transitions. The fairly high enthalpy change, even in the presence of such a diverse chemical mixture, suggests that ceramides can readily participate in the formation of a highly ordered lipid matrix. Thus, the physical chemical properties of isolated ceramides support the contention that ceramides have a pivotal role in controlling the mechanical and permeability properties of the stratum corneum of the skin.

Finally, the results from this study have implications for investigations into the role of ceramides in the function of the stratum corneum (14–18,33). The replacement of dipalmitoyl phosphatidylcholine by isolated stratum corneum ceramides in mixtures of cholesterol, 2-^{2}H-palmitic acid, and cholesterol sulphate led to the appearance of an isotropic peak in the ^{2}H NMR spectrum (14). These isolated ceramides would contain about 40% nonhydroxy fatty acids and 20% hydroxy fatty acids. Another 10% are the unusual ω-acyloxy ceramide, and the remainder are ceramides with the trihydroxy, phytosphingosine base (2–4). Because the dihydroxy ceramide, NFAC, readily formed a nonlamellar phase, the nonhydroxy fatty acid–containing ceramides fraction of the stratum corneum may energetically contribute to the formation of the isotropic phase.

For the studies involving model lipid mixtures, it is important to consider the composition of the ceramides of the skin and the model system. The bulk of the ceramides in the stratum corneum contain three or more hydroxy moieties in the head group. With respect to the fatty acid composition of the stratum corneum, less can be said. It appears that even in the most careful studies either the presence of hydroxylation or the presence of unsaturation was not determined (2–4). However, the most common acyl chainlengths are 16, 24 and 26 carbons. For model skin lipid mixtures, a number of different ceramide fractions have been used including type III and type IV bovine brain ceramides and synthetic palmitoyl sphingosine (14,15). Type III ceramides are obtained from enzymatic cleavage of sphingomyelin and do not contain any hydroxy fatty acids. The fatty acid composition of type III ceramide is not too different from the cerebrosides in that stearic acid and nervonic acid are prevalent. Type IV are derived from bovine brain cerebrosides and thus are comparable to the HFAC used in this study. Again, stearic and especially lignoceric and nervonic acids are present in appreciable quantities.

Although ceramides from the stratum corneum obviously are the most relevant for *in vitro* studies, the low abundance and difficulty in labeling severely limits spectroscopic investigations. It would seem that the use of a mixture of type III and type IV bovine brain ceramides would lead to the closest mimic of the stratum corneum ceramides (33). However, because the stratum corneum ceramides also contain phytosphingosine base, the average number of hydroxy groups of this mixture will be less than that of the stratum corneum. In addition, the brain ceramides have 60% of their acyl chains of either 18 or 24 carbons, while the stratum corneum appears to be more evenly distributed and with longer chains. Thus, there is perhaps greater asymmetry in the acyl chains of the stratum corneum ceramides in comparison to bovine brain model systems.

A modified procedure for obtaining hydroxy fatty acid and nonhydroxy fatty acid ceramides has been used for the study of the thermotropic behavior of ceramides. Whereas HFAC has a time-dependent enthalpy of transition, NFAC has a more complicated thermogram due to the ease in which it enters a metastable state. Mixtures of NFAC and HFAC have simple thermal properties for most compositions. Only with pure NFAC was a transition to a nonbirefringent liquid observed, indicating the profound effect of the introduction of a single hydroxy moiety on the resulting phase. The metastability of NFAC and time-dependent enthalpy of HFAC are qualitatively similar to NFA–CER and HFA–CER, respectively. This has the implication that the common functional groups of the ceramides and cerebrosides control the phase behavior of these lipids. Finally, the marked difference in the behavior of HFAC and NFAC allows for greater possibilities for the function of ceramides in the stratum corneum.

ACKNOWLEGDMENTS

We acknowledge the suggestions of Dr. Amir Salmon in improving the lipid isolation. We also appreciate the preliminary DSC studies carried out by Vincent Wong and Audrey Cheng and the assistance of Darasa Thumchai in performing the acyl chain analysis. We thank the reviewers for providing excellent comments and suggestions. Work was supported by a Grant-in-Aid from the University of Minnesota Graduate School and a gift from 3M Biosciences Laboratory.

REFERENCES

1. Bergelson, L.D. (1980) *Lipid Biochemical Preparations*, pp. 113–124, Elsevier, New York.
2. Lampe, M.A., Burlingame, A.L., Whitney, J., Williams, M.L., Brown, B.E., Roitman, E., and Elias, P.M. (1983) *J. Lipid Res. 24*, 120–130.
3. Lampe, M.A., Williams, M.L., and Elias, P.M. (1983) *J. Lipid Res. 24*, 131–140.
4. Wertz, P.W., and Downing, D.T. (1983) *J. Lipid Res. 24*, 759–765.
5. Williams, M.C., and Elias, P.M. (1982) *CRC Crit. Rev. Therap. Drug Carrier Syst. 3*, 95–122.
6. Elias, P.M., Cooper, E.R., Korc, A., and Brown, B.E. (1981) *J. Invest. Dermatol. 76*, 297–301.
7. Elias, P.M., Goerke, J., and Friend, D.S. (1977) *J. Invest. Dermatol. 69*, 535–546.
8. Wertz, P.W., and Downing, D.T. (1982) *Science 217*, 1261–1262.

9. Karlsson, K-A., and Pascher, I. (1971) *J. Lipid Res. 12*, 466–472.
10. Pascher, I. (1976) *Biochim. Biophys. Acta 455*, 433–451.
11. Lofgren, H., and Pascher, I. (1977) *Chem. Phys. Lipids 20*, 273–284.
12. Pascher, I., and Sundell, S. (1977) *Chem. Phys. Lipids 20*, 175–191.
13. Wertz, P.W., Swartzendruber, D.C., Kitko, D.J., Madison, K.C., and Downing, D.T. (1989) *J. Invest. Dermatol. 93*, 169–172.
14. Abraham, W., and Downing, D.T. (1991) *Biochim. Biophys. Acta 1068*, 189–194.
15. Fenske, D.B., Thewalt, J., Bloom, M., and Kitson, N. (1994) *Biophys. J. 67*, 1562–1573.
16. Ongpipattanakul, B., Francoeur, M.L., and Potts, R.O. (1994) *Biochim. Biophys. Acta 1190*, 115–122.
17. Bouwstra, J.A., de Vries, M.A., Gooris, G.S., Bras, W., Brussee, J., and Ponec, M. (1991) *J. Control. Rel. 15*, 209–220.
18. Mattai, J., Froebe, C.L., Rhein, L.D., Simion, F.A., Ohlmeyer, H., Su, D.T., and Friberg, S.E. (1993) *J. Soc. Cosmet. Chem. 44*, 89–100.
19. Radin, N.S. (1976) *J. Lipid Res. 17*, 290–293.
20. Carter, H.E., Rothfus, J.A., and Gigg, R. (1961) *J. Lipid Res. 2*, 228–234.
21. Wiedmann, T.S., and Salmon, A. (1991) *Lipids 26*, 364–368.
22. Mabrey, S., and Sturtevant, J.M. (1976) *Proc. Natl. Acad. Sci. USA 73*, 3862–3870.
23. O'Brien, J.L., and Rouser, G. (1964) *J. Lipid Res. 5*, 339–347.
24. Shah, J., Atienza, J., Rawlings, A.V., and Shipley, G.G. (1994) *Biophys. J. 66*, A288.
25. Curatolo, W. (1982) *Biochemistry 21*, 1761–1764.
26. Curatolo, W., and Jungalwala, F.B. (1985) *Biochemistry 24*, 6608–6613.
27. Wu, W-G., Chong, P.L-G., and Guang, C-H. (1985) *Biophys. J. 47*, 237–242.
28. White, S.H., Mirejovsky, D., and King, G.I. (1988) *Biochemistry 27*, 3725–3732.
29. Ruocco, M.J., Atkinson, D., Small, D.M., Skarjune, R.P., Oldfield, E., and Shipley, G.G. (1981) *Biochemistry 20*, 5957–5966.
30. Bunow, M.R. (1979) *Biochim. Biophys. Acta 574*, 542–546.
31. Jackson, M., Johnston, D.S., and Chapman, D. (1988) *Biochim. Biophys. Acta 944*, 497–506.
32. Johnston, D.S., and Chapman, D. (1988) *Biochim. Biophys. Acta 939*, 603–614.
33. Rehfeld, S.J., Plachy, W.Z., Williams, M.L., and Elias, P.M. (1988) *J. Invest. Dermatol. 91*, 499–505.

[Received June 6, 1994, and in revised form December 8, 1994; Revision accepted December 28, 1994]

Analysis of Lipoxygenase Kinetics by High-Performance Liquid Chromatography with a Polymer Column

Alberto Nuñez* and George J. Piazza

ERRC, ARS, USDA, Wyndmoor, Pennsylvania 19118

ABSTRACT: Soybean lipoxygenase (LOX; EC 1.12.11.12) catalyzes the oxygenation of polyunsaturated fatty acids, acylglycerols and phosphoglycerols, producing a regio- and enantiospecific hydroperoxide product. The goal of this work was to measure the relative rate of LOX-catalyzed oxidation of mixtures of lipids containing linoleate, using high-performance liquid chromatography (HPLC) and a light-scattering detector (LSD). Previous literature suggested that reversed-phase HPLC with silica-based columns could be used for the separation of individual fatty acids, acylglycerols, phosphoglycerides and their oxidation products. However, these columns produced ineffective separations of phosphoglycerides unless choline chloride and a strong base, such as KOH, are present in the mobile phase. Such modifiers precluded the use of the LSD. It was found that a reversed-phase column based upon an organic polymer support, rather than on silica, was able to separate these mixtures with a ternary solvent gradient of methanol/water/acetonitrile without the need for the addition of modifiers. The oxidation time course of a mixture of linoleic acid, trilinolein and 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine was followed using the developed HPLC method. The results showed that trilinolein and phosphatidylcholine reacted at one-tenth the rate of linoleic acid. The diacylglycerol, 1,3-dilinolein, was oxidized at a rate that was approximately 40% that of linoleic acid, with the formation of mono- and dihydroperoxides as well as other unidentified products.

Lipids 30, 129–133 (1995).

The enzyme, lipoxygenase (LOX; EC 1.13.11.12), catalyzes the oxidation of polyunsaturated fatty acid containing a *cis, cis*-1-4-pentadiene moiety, producing fatty acid hydroperoxides. It is known that LOX from a variety of natural sources produces a single major product (1,2). For example 13(*S*)-hydroperoxy-9,11-octadecadienoic acid results from the action of soybean LOX upon linoleic acid (LA) (3). Recently it was shown that immobilization of LOX enhances its stability, thereby increasing its potential as a synthetic reagent for the selective oxidation (4,5). In addition to free fatty acid substrates, early studies showed that polyunsaturated acylglycerols undergo oxidation with soybean LOX (6–8). More recently, it was shown that this enzyme catalyzes the oxidation of phosphatidylcholine, provided that a bile salt surfactant is present (9). Structural analysis of the products illustrated the high positional and stereochemical specificity of this reaction (10). Although prior work has shown that LOX acts upon fatty acids, acylglycerols and phosphoglycerides, the substrate specificity of LOX has never been determined with a bile salt present in the reaction medium.

To measure enzyme specificity, Schellenberger *et al.* (11) have developed a statistical method for the analysis of reactions of competitive substrates. To use this method, the relative concentrations of all substrates or products must be followed over time by an analytical technique. For the analysis of LOX substrates and products, high-performance liquid chromatography (HPLC) is an excellent option. There is a vast volume of HPLC methodologies published for the separation of neutral and polar acylglycerols using normal-phase (NP) and silica-based reversed-phase (RP) columns (12). Although NP-HPLC is used for the separation of lipid classes, separation of the components of each class is usually accomplished by RP-HPLC. Fatty acids and neutral acylglycerols can be separated with an RP-HPLC method that uses a quaternary stepped mobile phase gradient (13). However, the successful separation of phosphoglycerides requires the inclusion of a modifying salt in the mobile phase. For example, an RP-HPLC method described by Patton *et al.* (14) used 20 mM choline chloride in methanol/water/acetonitrile as the mobile phase. More recently, it was shown that inclusion of ammonium acetate in the mobile phase greatly increased peak sharpness (15,16).

For monitoring the elution of acylglycerols and phosphoglycerides ultraviolet (UV) detection at 205 nm is often used. However, there are two significant disadvantages to the use of a UV detector. First, solvents with significant absorption above 200 nm cannot be used in the mobile phase and, second, the degree of unsaturation in the sample strongly affects detector sensitivity. A detector that does not have these deficiencies is the light-scattering detector (LSD). On the other hand, its use is limited when salts or buffers are used in the mobile phase. Although 0.1% ammonium acetate may be

*To whom correspondence should be addressed at ERRC, ARS, USDA, Wyndmoor, PA 19118.

Abbreviations: 1,3-DL, 1,3-dilinolein; DO, 1,3-diolein; HPLC, high-performance liquid chromatography; LA, linoleic acid; LSD, light-scattering detector; 1L-2S-PC, 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; LOX, lipoxygenase; NP, normal phase; 1P-2Ll-PE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; RP, reversed phase; TL, trilinolein; UV, ultraviolet.

Copyright © 1995 by AOCS Press

used with an LSD, the addition of choline chloride results in unacceptable baseline noise. It occurred to us that a polymer-based RP column would adsorb the polar functionalities of phosphoglycerides less tightly than a silica-based RP column, alleviating the necessity for the addition of mobile phase salts and buffers. This would be an important advance, as it would allow the utilization of an LSD for the quantitation of all lipids species. Therefore, a polymer-based RP-HPLC method was developed for the analysis of mixtures of free LA, phosphoglycerides and acylglycerols containing linoleate using a mobile phase that was free of salt. This method was utilized to determine the relative rate of oxidation by LOX of these lipid classes.

MATERIALS AND METHODS

Materials. Soybean LOX (lipoxidase, Type 1-B), LA, trilinolein (TL), 1,3-dilinolein (1,3-DL) and 1,3-diolein (DO) were purchased from Sigma (St. Louis, MO). Avanti Polar Lipids, Inc. (Alabaster, AL) supplied 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (1P-2L-PE) and 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (1L-2S-PC). All HPLC solvents (hexane, acetonitrile and methanol) were obtained from Burdick and Jackson (Muskegon, MI). Water was purified to a resistance of 18 MΩ-cm using a Barnstead (Dubuque, IA) NANOpure system. All other reagents were of the highest purity available.

Formation of hydroperoxide. An aliquot of the substrate (6 μmol linoleate) and 2 μmol of DO (as internal standard) dissolved in chloroform was placed in a 10-mL Erlenmeyer flask, and the solvent removed under a stream of dry nitrogen. In addition to the substrate, the reaction medium contained 0.2 mg LOX, 0.2 mL 100 mM deoxycholate and 1.8 mL aqueous buffer, consisting of an equal 0.1 M mixture of the buffers AMP, TRICINE, HEPES and MES (respectively, 2-amino-2-methyl-1-propanol hydrochloride, N-tris(hydroxymethyl)-methylglycine, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid, and 2-N-morpholino)ethanesulfonic acid). Oxidation was conducted at 15°C with agitation at 250 rpm for an indicated time. Reactions were quenched with 400 μL 1M citric acid and extracted within 1 h with two 1.5 mL aliquots of chloroform/methanol (2:1, vol/vol). The solvent was removed under a stream of nitrogen, and the sample was redissolved in 0.5 mL of methylene chloride.

HPLC. Liquid chromatographic analysis was performed with a Hewlett-Packard (Palo Alto, CA) Model 1050 HPLC equipped with a gradient program and autosampler. Detection of analytes was by a variable wavelength UV detector, HP 1050 Series, connected in series with a Varex IIA Universal Evaporative Light-Scattering Detector (ELSD, Rockville, MD) operated at 100°C with nitrogen (40 psi) as the nebulizer gas. All analyses were accomplished with a HEMA-RP C18 10 micron column (250 mm × 4.6 mm) at programmed flow rate (1 to 1.5 mL/min). Also, the studies included an ADSORBOSPHERE C18 5-micron (250 mm × 4.6 mm) column. Both columns were obtained from Alltech (Deerfield, IL). Other types of silica-based columns (CHROMSPHER Si, 5μ, 200 mm × 3 mm, and Chrompack Chromsep 5μ LICHROSORB DIOL, 5μ, 200 mm × 3 mm, both supplied by Chrompack, Raritan, NJ) were evaluated but were found to give inferior separations.

Time course and relative rate analysis. A typical reaction time course consisted of seven time points taken at five-minute intervals. The data for each time point was derived from a separately prepared reaction mixture. Each data point represents the mean of at least three injections of 20 μL of the reaction extract. Oxidations were followed by monitoring with both a UV detector (205 nm) and an LSD. Comparable results were obtained with both detectors. The analysis of the data from the mixtures of substrates to obtain the relative rate of oxidation was accomplished using the computer program developed by Schellenberger *et al.* (11).

RESULTS AND DISCUSSION

HPLC analysis. Preliminary studies on the separation of a mixture of free fatty acid, acylglycerols and phosphoglycerides were made with a silica-based ADSORBOSPHERE RP column. Although several gradient shapes, and combinations of solvents (e.g., isopropanol, acetonitrile, methanol, 1% ammonium acetate in methanol, water) were tried, phosphoglycerides eluted as significantly tailed peaks, and quantitation was not possible. Addition of 20 mM choline chloride to the mobile phase reduced tailing noticeably, as described previously (10,14). However, it was found that the concentration of choline chloride in the mobile phase could be reduced to 1 mM if the column was first equilibrated with 20 mM choline chloride for 1–2 h. Total removal of this modifier resulted in slowly increasing peak tailing of the phosphoglyceride with time. In addition to choline chloride, it was found that the addition of base was needed to obtain completely symmetrical phosphoglyceride peaks. Trimethylamine, ammonium hydroxide and KOH were tested as bases, and the best performance was obtained with 5 mM KOH as a component of the elution system. The best mobile phase gradient that was found is shown in Table 1. Using this gradient, the

TABLE 1
Mobile-Phase Gradient and Flow Rate Program for Separation of Linoleic Acid, 1-Palmitoyl-2-Linoleoyl-*sn*-Glycerol-3-Phosphoethanolamine (1P-2L-PE), 1-Linoleoyl-2-Stearoyl-*sn*-Glycero-3-Phosphocholine (1L-2S-PC) and Trilinolein on an ADSORBOSPHERE C18 Column[a]

Time (min)	Composition of mobile phase[b]			Flow rate (mL/min)
	Eluant A[c]	Eluant B[d]	Eluant C[e]	
0	95	5	0	0.5
4	95	5	0	0.8
14	95	5	0	1.9
16	88	5	7	1.9

[a]From Alltech (Deerfield, IL). [b]Composition in % (vol/vol). [c]Eluant A: 5 mM KOH in methanol. [d]Eluant B: 20 mM choline chloride in methanol. [e]Eluant C: hexane.

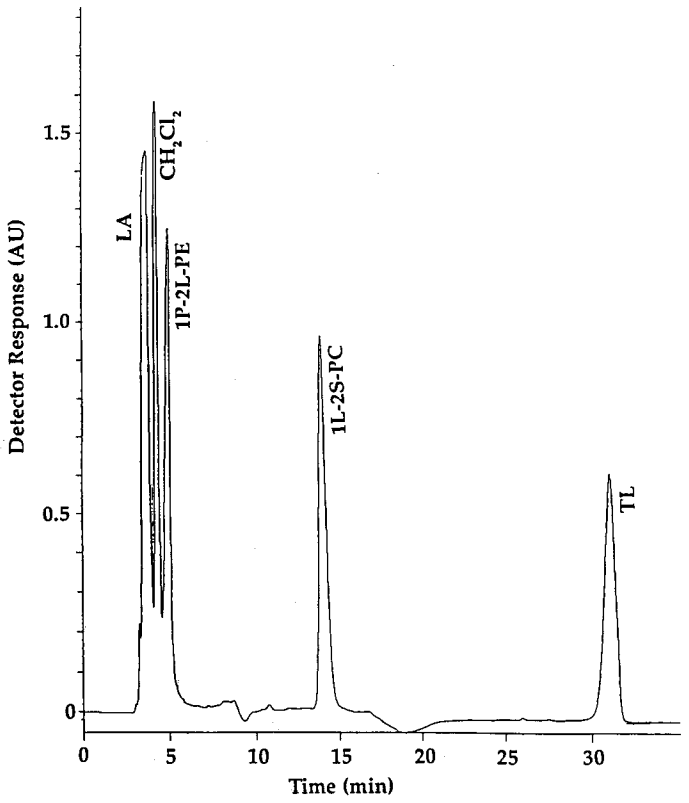


FIG. 1. Elution profile of the injection of 20 µL of a mixture of linoleic acid (LA), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (1P-2L-PE), 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (1L-2S-PC) and trilinolein (TL) (2 mg/mL in methylene chloride) with silica-based reversed-phase high-performance liquid chromatography using the solvents and modifiers described in Table 1. An Alltech (Deerfield, IL) 5 micron ADSORBOSPHERE C18 column (250 mm × 4.6 mm) was used, with ultraviolet detection at 205 nm.

separation of LA, 1P-2L-PE, 1L-2S-PC and TL was attempted. The elution profile is shown in Figure 1, and it can be seen that the peaks corresponding to the LA, the injecting solvent, methylene chloride and 1P-2L-PE were not satisfactorily resolved. Due to the presence of an unsatisfactory level of baseline noise in the LSD, only the UV detector was used. The inclusion of KOH in the mobile phase also rapidly cleaves the organic layer from the silicate particles and causes damage to the silica structure of the column, decreasing its resolution and lifetime (17). For these reasons this method was abandoned. Other silica-based columns (CHROMSPHER Si, 5µ, and LICHROSORB DIOL, 5µ) were also tested but were found to be unsatisfactory for the separation of this mixture of components.

Because of problems encountered with the silica-based RP column, a polymer-based (hydroxyethyl methacrylate/dimethacrylate copolymer) HEMA-RP C18 column was evaluated. This column is resistant to bases and lacks the interactions associated with the free silanols in the silica-based RP column. After trying different mobile phases, the best separation was achieved with a ternary gradient of water/methanol/acetonitrile, without the need for inclusion of salt, buffer or base (Table 2). Figure 2 shows the chromatogram for the separation of the mixture of LA, 1P-2L-PE, 1L-2S-PC, 1,3-DL, DO and TL with the HEMA-RP C18 column. All components, including phosphoglycerides, eluted as sharp peaks. The solvent gradient shown in Table 2 was used for elution and detection was by the LSD.

TABLE 2
Mobile-Phase Gradient and Flow Rate Program for Separation of Linoleic Acid, 1P-2L-PE, 1L-2S-PC and Trilinolein on an HEMA-RP C18 Column[a]

Time (min)	Composition of mobile phase[b]			Flow rate (mL/min)
	Eluant A[c]	Eluant B[d]	Eluant C[e]	
0	86	14	0	1.0
5	90	10	0	1.0
8	90	10	0	1.5
10	65	5	30	1.5
13	48	1	51	1.5

[a]Company source and abbreviations as in Table 1. [b]Composition in % (vol/vol). [c]Eluant A: methanol. [d]Eluant B: water. [e]Eluant C: acetonitrile.

Comparative kinetic study. A mixture of LA, 1L-2S-PC and TL was prepared in the presence of 10 mM deoxycholate and was subjected to the action of LOX. The concentrations of substrates over time were determined by HPLC using both

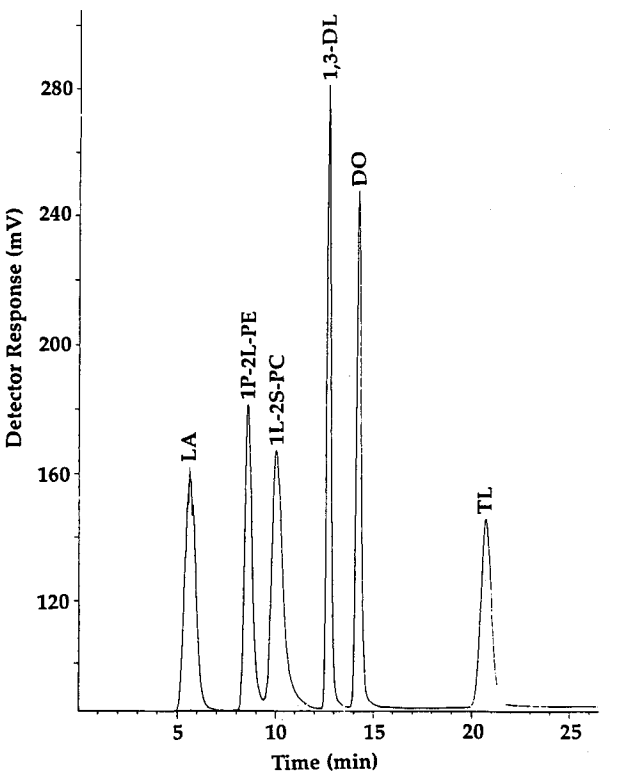


FIG. 2. Elution profile of the injection of 20 µL of mixture of LA, 1P-2L-PE, 1L-2S-PC, 1,3-dilinolein (1,3-DL), 1,3-diolein (DO) and TL (2 mg/mL in methylene chloride) with polymer-based reversed-phase high-performance liquid chromatography using the solvents described in Table 2. An Alltech 10 micron HEMA-RP C18 column (250 mm × 4.6 mm) was used, with light-scattering detection. Abbreviations and company source as in Figure 1.

the LSD and the UV detector. However, at the longer time points the concentration of LA became very low, and only the UV detector was sensitive enough to measure the remaining LA. Figure 3 shows the chromatogram of the substrates and hydroperoxides formed from a mixture of LA, 1L-2S-PC and TL after 30 min of reaction. Peaks B, D and G correspond to LA, 1L-2S-PC and TL, respectively. Peaks A, C and F are the corresponding hydroperoxide products. Peak E is the internal standard (DO). The assignment of each hydroperoxide was made by oxidation of each substrate separately. Figure 4 shows a typical time course of LOX-catalyzed oxidation of LA, 1L-2S-PC and TL. The relative rate of oxidation of the multiple substrates was calculated by the method of Schellenberger *et al.* (11), and the results are presented in Table 3. As expected, LA was oxidized at the fastest rate, and the rates of oxidation of 1L-2S-PC and TL were only 10% of that of LA. Control experiments showed that nonenzymatic oxidation did not occur over the time-span of the experiment.

The competitive oxidation of LA and 1,3-DL by LOX was also monitored by HPLC. Surprisingly, 1,3-DL was oxidized at a rate that was 40% as fast as LA (Table 3). The relatively high rate of oxidation of 1,3-DL as compared to TL and 1L-2S-PC suggests that steric factors strongly influenced the ability of LOX to promote hydroperoxide formation. The oxidation of 1,3-DL by LOX led to the formation of mono- and dihydroperoxides as major products, with small amounts of other unidentified products as determined by HPLC.

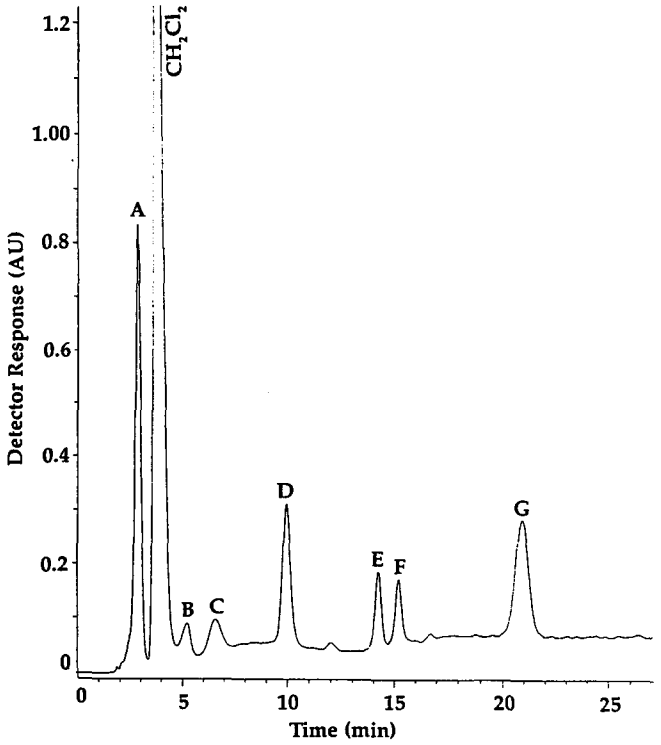


FIG. 3. Elution profile of a mixture of LA, 1L-2S-PC and TL after 30 min of incubation with lipoxygenase, with polymer-based reversed-phase high-performance liquid chromatography using the solvents described in Table 2. An Alltech 10-micron HEMA-RP C18 column (250 mm × 4.6 mm) was used, with ultraviolet detection at 205 nm. A, peroxide of LA (B); C, peroxide of 1L-2S-PC (D); E, DO (internal standard); F, peroxide of TL (G). Abbreviations and company source as in Figures 1 and 2.

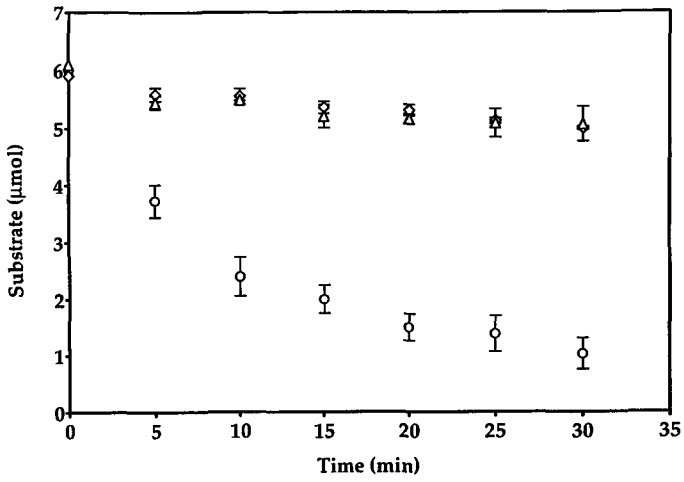


FIG. 4. Time course of the oxidation by lipoxygenase of a mixture of LA (○); TL (◇) and 1L-2S-PC (△), as determined by reversed-phase high-performance liquid chromatography. The chromatographic conditions are described in Table 2. Abbreviations as in Figure 1.

TABLE 3
Relative Rate of Oxidation of Linoleic Acid (LA), 1L-2S-PC, Trilinolein (TL) and 1,3-DL by Lipoxygenase[a]

Substrates	Relative rate (%)[b]
LA	100
1L-2S-PC	11 ± 1
TL	10 ± 3
LA	100
1,3-DL	36 ± 2

[a]Each determination consisted of seven samples containing 6 µmol linoleate for each substrate, 10 mM deoxycholate, and 0.2 mg lipoxygenase at pH 8 and 15°C. Samples were analyzed every 5 min from time 0 to 30. Abbreviations as in Table 1.
[b]The data are the means ± SD (n = 6).

In conclusion, the use of the HPLC with a polymer-based column allowed for the separation and quantitation of a mixture of free fatty acid, acylglycerols and phosphoglycerides without the need of modifiers in the mobile phase. Detection by both LSD and UV can be made. The utility of this HPLC method was demonstrated using chemically pure substrates. However, this method should be useful for analyzing the extent of oxidation in many fats and oils.

REFERENCES

1. Hamberg, M., and Samuelsson, B. (1965) *Biochem. Biophys. Res. Commun. 21,* 431–536.
2. Holman, R.T., Egwim, P.O., and Christie, W.W. (1969) *J. Biol. Chem. 244,* 1149–1151.
3. Hamberg, M. (1971) *Analytical Biochemistry 43,* 515–526.
4. Parra-Diaz, D., Brower, D.P., Medina, M.B., and Piazza, G.J. (1993) *Biotechnol. Appl. Biochem. 18,* 359–367.

5. Piazza, G.J., Brower, D.P., and Parra-Diaz, D. (1994) *Biotechnol. Appl. Biochem. 19,* 243–252.
6. Koch, R.B., Stern B., and Ferrari, C.G. (1969) *Arch. Biochem. Biophys. 78,* 165–179.
7. Guss, P.L., Richardson, T., and Stahmann, M.A. (1968) *J. Am. Chem. Soc. 45,* 272–276.
8. Christofer, J.P., Pistorius, E., and Axelrod, B. (1970) *Biochem. Biophys. Acta 198,* 12–19.
9. Eskola, J., and Laakso, S. (1983) *Biochem. Biophys. Acta 751,* 305–311.
10. Brash, A.R., Ingram, C.D., and Harris, T.M. (1987) *Biochemistry 26,* 5465–5471.
11. Schellenberger, V., Siegel, R.A., Rutter, W. (1993) *Biochemistry 32,* 4344–4348.
12. Christie, W.W. (1985) *Z. Lebensm.-Unters Forsch. 181,* 171–182.
13. Antonoppoulou, S., Andrikopoulos, N.K., and Demopoulos, C.A. (1994) *J. Liquid Chromatogr. 17,* 633–648.
14. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1982) *J. Lipid. Res. 23,* 190–196.
15. Therond, P., Couturier, M., Demelier, J.F., and Lemonnier, F. (1993) *Lipids 28,* 245–249.
16. van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz, E.A. (1985) *J. Free Radicals Biol, Med. 1,* 215–225.
17. Wehrli, A., Hildenbrand, J.C., Keller, H.P., and Stampfli, R. (1978) *J. Chromatogr. 149,* 199–210.

[Received September 8, 1994, and in revised form December 27, 1994; accepted January 9, 1995]

Effect of *d*-α-Tocopherol Analogues on Lipoxygenase-Dependent Peroxidation of Phospholipid–Bile Salt Micelles

Hirofumi Arai[a], Akihiko Nagao[b], Junji Terao[b,*], Tetsuya Suzuki[a] and Kozo Takama[a]

[a]Department of Food Science and Technology, Faculty of Fisheries, Hokkaido University, Hokkaido 041, Japan and [b]National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Ibaraki 305, Japan

ABSTRACT: In order to know whether or not vitamin E acts as an effective antioxidant in lipoxygenase-dependent peroxidation of phospholipids, the effect of vitamin E and vitamin E analogues, 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), was investigated in enzymatic lipid peroxidation of bile salt micelles of pig liver phosphatidylcholine (PC) using soybean lipoxygenase. 15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid was exclusively produced by the reaction with the PC molecular species containing arachidonic acid moiety, indicating that the hydroperoxidation of pig liver PC entirely progresses through the enzymatic reaction. PMC suppressed the accumulation of PC-hydroperoxides (PC–OOH) more efficiently than either *d*-α-tocopherol (α-Toc) or Trolox C, and 50% inhibition concentration by PMC was close to that of quercetin, a known lipoxygenase inhibitor from natural origin. The antioxidant activity of PMC was also superior to that of either α-Toc or Trolox C in ferrous ion-induced nonenzymatic oxidation of PC micelles in the presence of a trace amount of PC–OOH, although the radical-scavenging activities of these compounds in solution were similar or comparable to one another. In conclusion, PMC is more effective than α-Toc as an inhibitor of lipoxygenase reaction with phospholipids and of autoxidation in phospholipids. The phytyl chain of α-Toc seems to be unfavorable for exerting an inhibitory effect on lipoxygenase reaction with phospholipid–bile salt micelles.
Lipids 30, 135–140 (1995).

It has been suggested that lipid peroxidation is one of the significant pathophysiological factors in the oxidative stress leading to various diseases, including atherosclerosis (1,2). Although a large number of studies have been carried out using a variety of model systems, the mechanism of lipid peroxidation occurring *in vivo* is still not entirely clarified. In particular, the mechanism of its initial reaction remains equivocal in many cases. In recent years, mammalian lipoxygenase (EC 1.13.11.12) has received much attention as a possible initiator for propagating lipid peroxidation in the process of oxidative modification of low-density lipoprotein (3) and biomembranes (4,5).

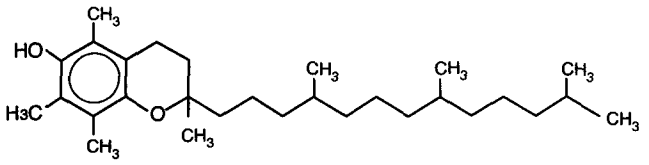
Human tissues possess a well-organized defense system against oxidative stress (6), and *d*-α-tocopherol (α-Toc) is considered to act as a primary antioxidant in the suppression of the propagation reaction of nonenzymatic lipid peroxidation by trapping chain-propagating lipid peroxyl radicals (7). On the other hand, it has also been reported that α-Toc plays an important role in the modulation of lipoxygenase pathway in arachidonic acid metabolism (8,9). Thus, it is of much interest to know the antioxidant activity of α-Toc against lipoxygenase-dependent lipid peroxidation, when this enzymatic reaction is involved in the initiation of propagating lipid peroxidation *in vivo*.

We studied whether or not α-Toc acts as an effective inhibitor of the enzymatic peroxidation of unsaturated phospholipids using soybean lipoxygenase, because soybean lipoxygenase can oxidize phospholipids directly in the presence of bile salt (10,11). Also, phospholipids are recognized as a major target of oxidative damage induced by lipid peroxidation. The role of the phytyl side chain on the antioxidant activity of α-Toc has been frequently discussed in peroxidation of membranous phospholipids (12–15). Thus, the inhibitory effects of α-Toc and α-Toc analogues [**1**], 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) [**2**] and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C) [**3**] (Scheme 1), on this enzymatic reaction system were compared to those of nonenzymatic ferrous ion-induced phospholipid peroxidation in order to understand the role of the phytyl side chain in the inhibition. The results strongly suggest that an α-Toc analogue, PMC, is superior to α-Toc in the inhibition of lipoxygenase-dependent phospholipid peroxidation.

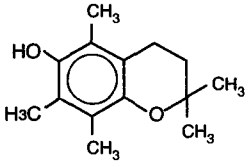
*To whom correspondence should be addressed at National Food Research Institute, MAFF, Kannondai, Tsukuba, Ibaraki 305, Japan.

Abbreviations: AMVN, 2,2′-azobis-(2,4-dimethylvaleronitrile); BHA, butylated hydroxyanisole [2(3)-*tert*-butyl-4-methoxyphenol]; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibition concentrations; MeL-OOH, methyl linoleate hydroperoxides; PC, phosphatidylcholine; PC–OOH, phosphatidylcholine hydroperoxides; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychroman; SA–PC, 1-stearoyl-2-arachidonyl phosphatidylcholine; TBARS, thiobarbituric acid reacting substances; α-Toc, *d*-α-Tocopherol; Trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UV, ultraviolet.

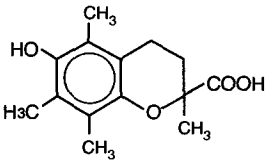
Copyright © 1995 by AOCS Press



[1]



[2]



[3]

SCHEME 1

MATERIALS AND METHODS

Materials. Soybean lipoxygenase (Type I-B, 94,000 units/mg protein) and 1-stearoyl-2-arachidonyl phosphatidylcholine (SA–PC; 99%) were purchased from Sigma (St. Louis, MO). α-Toc and PMC were supplied by Eisai Pharmaceutical Co. (Tokyo, Japan). Trolox C was purchased from Aldrich (Milwaukee, WI). β-Carotene was purchased from Merck (Darmstadt, Germany). Butylated hydroxyanisole [2(3)-*tert*-butyl-4-methoxyphenol; BHA] and caffeic acid (3,4-dihydroxycinnamic acid) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Quercetin was obtained from Wako Pure Chem. (Osaka, Japan). Pig liver phosphatidylcholine (PC) was purchased from Funakoshi Co. (Tokyo, Japan) and purified by reversed-phase column chromatography (16). The fatty acid composition of pig liver PC was determined by gas chromatography after transmethylation as follows: 16:0, 27.6%, 18:0; 17.0%, 18:1; 27.5%, 18:2; 18.0%, 20:4; 7.7%. PC–hydroperoxides (PC–OOH) were prepared from egg yolk PC (Sigma; 99%) by photooxidation (17) and purified by reversed-phase column chromatography (16). All other chemicals and solvents were of analytical grade.

Preparation of PC micelles. Pig liver PC (1 μmol) in chloroform solution and an appropriate amount of antioxidant in a methanol solution were mixed in a round-bottom test tube and evaporated to a thin lipid film under nitrogen and finally *in vacuo*. The lipid film was rapidly dispersed in 900 μL HEPES buffer (20 mM, pH 7.4) containing 20 mM sodium deoxycholate by vigorous shaking on a vortex mixer for 1 min and sonicated with a BRANSON B-12 ultrasonifier (55 kHz) for 1 min.

Lipoxygenase reaction of PC micelles. Lipid peroxidation of PC micelles was induced by the addition of 2.8×10^4 units of soybean lipoxygenase in 100 μL HEPES buffer (20 mM, pH 7.4). The mixture was incubated with continuous shaking at 37°C in the dark. At regular intervals, PC–OOH in the mixture was determined by reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection according to the method reported previously (18), in which the mixture of methanol/water (95:5, vol/vol) was used as an eluting solvent.

HPLC analysis of the products obtained from the lipoxygenase reaction with SA–PC. SA–PC micelles without antioxidants were prepared and oxidized by soybean lipoxygenase for 60 min in the same condition described above. The lipid fraction was extracted from the reaction mixture and was treated with phospholipase A_2 from bee venom by the method of Hughes *et al.* (19). Then the extracted products were analyzed by reversed-phase HPLC with UV detection according to the method reported previously (20). Authentic 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) was prepared by enzymatic oxidation of arachidonic acid using soybean lipoxygenase (21). The isomeric mixture of HPETE was prepared from autoxidized arachidonic acid (20).

Nonenzymatic oxidation of PC micelles induced by ferrous ion–ascorbate system. PC micelles with or without antioxidants were prepared by the same method as described in the preparation for lipoxygenase reaction, except for the addition of PC–OOH before preparation of the micelles. The lipid peroxidation was induced by the addition of $FeSO_4$ dissolved in an aqueous solution of ascorbic acid. The assay mixture consisted of 1 mM PC, 0.01 mM PC–OOH, appropriate concentration of antioxidant, 0.1 mM $FeSO_4$, 1 mM ascorbic acid and 20 mM sodium deoxycholate in 20 mM HEPES buffer (pH 7.4). The mixture was incubated with continuous shaking at 37°C in the dark. At regular intervals, samples were subjected to thiobarbituric acid assay (22), and the amount of thiobarbituric acid reacting substances (TBARS) were expressed as μM malondialdehyde.

Measurement of peroxyl radical-scavenging activity in solution. Peroxyl radical-scavenging activities of α-Toc, PMC and Trolox C in solution were determined according to the method described previously (23). Briefly, 100 mM of methyl linoleate in a solution of *n*-hexane/2-propanol/ethanol (8:3:1, by vol) containing each antioxidant was subjected to radical chain oxidation by the addition of 10 mM of 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN) at 37°C in the dark. The resulting methyl linoleate hydroperoxides (MeL–OOH) were quantified by normal-phase HPLC technique (24). The rate of oxidation during the induction period (R_{inh}) and the rate of oxidation after the induction period, that is, the rate of propagation (R_p), were obtained from the slope of the curve of MeL–OOH formation. The apparent induction period (t_{inh}) could be calculated from the intersecting point of the curve for R_{inh} and that for R_p. We applied the method by Burton and Ingold (25) for the calculation of kinetic parameters of each antioxidant.

RESULTS

Effect of α-Toc and its analogues on lipoxygenase reaction. The bile salt micelles of PC were rapidly oxidized by soybean lipoxygenase, although neither its multilamellar liposomes nor large unilamellar liposomes were oxidized after incubation for 2 h as previously reported by Eskola and Laakso (data

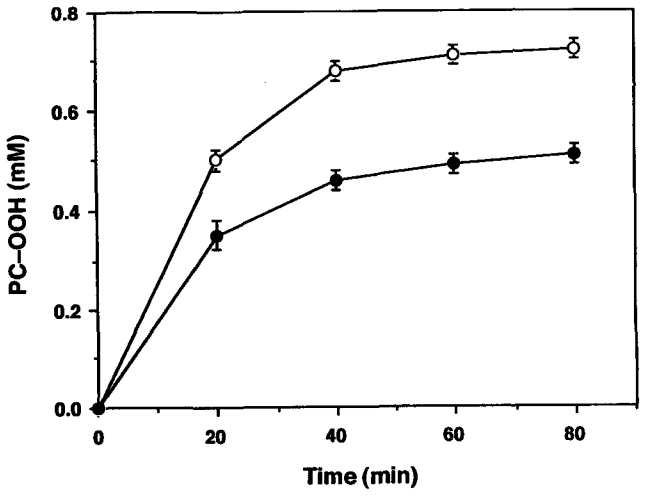


FIG. 1. Effect of α-Toc on lipoxygenase-dependent peroxidation of PC micelles. The concentration of PC–OOH was determined by reversed-phase HPLC with ultraviolet detection (Ref. 18). The reaction system consisted of pig liver PC (1 mM), soybean lipoxygenase (2.8×10^4 units) and 20 mM sodium deoxycholate in 20 mM HEPES buffer (1.0 mL, pH 7.4). No addition (○), 0.1 mM α-Toc (●). α-Toc, *d*-α-tocopherol; PC, phosphatidylcholine; PC–OOH, phosphatidylcholine–hydroperoxides; HPLC, high-performance liquid chromatography.

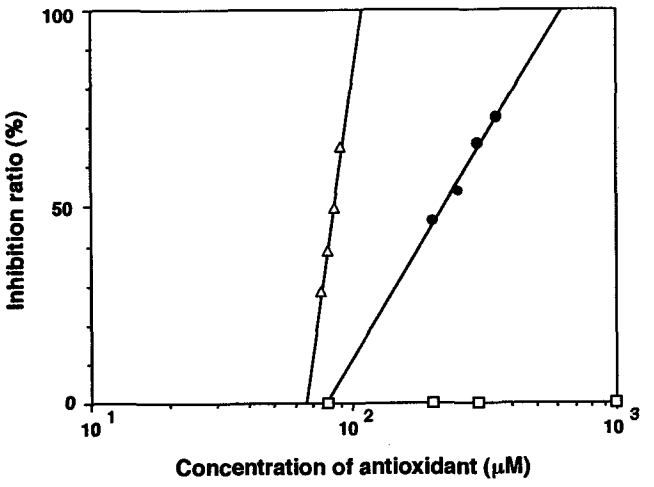


FIG. 2. The relationship between the inhibition by α-Toc (●), 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) (△), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (Trolox C) (□) on lipoxygenase reaction of PC micelles and their concentrations. The reaction system was the same as that described in the legend in Figure 1. Abbreviations as in Figure 1.

are not shown here) (10). Figure 1 shows the time course of the accumulation of PC–OOH by the reaction of PC micelles with soybean lipoxygenase in the presence and the absence of α-Toc. In the absence of α-Toc, PC–OOH accumulation progressed rapidly in the initial stage and terminated after 40 min incubation because of the exhaustion of substrate. Both the rate of PC–OOH accumulation and its maximum amount were suppressed in the presence of 0.1 mM α-Toc. Figure 2 shows the relationship between the concentration of α-Toc or α-Toc analogues and the inhibition ratio of PC–OOH accumulation after 60 min incubation. The 50% inhibition concentrations (IC_{50}) of α-Toc and PMC were 220 and 80 μM, respectively. Trolox C did not exert any inhibitory effect in the range from 80 μM to 1 mM. Inhibition ratio (%) of α-Toc, PMC and Trolox C were calculated from the following equation:

$$\text{inhibition ratio (\%)} = ([B] - [A])/[B] \times 100 \qquad [1]$$

where [*A*] and [*B*] are the concentration of PC–OOH after 60 min incubation in the presence of either α-Toc or α-Toc analogues (PMC and Trolox C) and that in the absence of them, respectively.

Effect of lipophilic antioxidants on lipoxygenase reaction. The inhibitory effect of some lipophilic antioxidants on the lipoxygenase reaction was also measured by the same procedure shown in the case of α-Toc and its analogues (Table 1). The IC_{50} of butylated hydroxyanisole (BHA) and quercetin were comparable to that of PMC. The inhibition by caffeic acid was weaker than that of α-Toc, whereas β-carotene showed no inhibitory effect even at the concentration of 1 mM.

Product specificity of lipoxygenase reaction. We investigated the product specificity of the reaction of PC micelles with lipoxygenase by using SA–PC to confirm that the reaction proceeds through enzymatic mechanism. It is known that autoxidation of SA–PC produces several isomers of HPETE at the arachidonic acid moiety (26). Figure 3 shows HPLC chromatograms of authentic 15-HPETE (A), phospholipase A_2-mediated hydrolyzed products from SA–PC after incubation with soybean lipoxygenase (B) and the isomeric mixture of HPETE from autoxidized arachidonic acid (C). The chromatogram of HPETE of SA–PC with lipoxygenase reaction was coincident with that of 15-HPETE. Thus, it was found that oxygen molecule exclusively attacked the 15-position of arachidonic acid moiety in the reaction of SA–PC with soybean lipoxygenase at neutral pH, similarly to the reaction at pH 9.0 reported by Brash *et al.* (11). Consequently, it seems that hydroperoxidation of pig liver PC in this reaction system also progresses through the enzymatic reaction.

Inhibition by α-Toc and its analogues on ferrous ion-dependent peroxidation in micelles. We investigated the effect

TABLE 1
The 50% Inhibition Concentrations (IC_{50}) of Antioxidants on Lipoxygenase Reaction of Phosphatidylcholine Micelles

Antioxidants	IC_{50} (μM)[a]
d-α-Tocopherol	220
PMC	80
Trolox C	no effect
β-Carotene	no effect
BHA	70
Quercetin	90
Caffeic acid	610

[a]IC_{50} of each antioxidant was determined by the procedure shown in Figure 3. BHA, butylated hydroxyanisole; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychroman; Trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

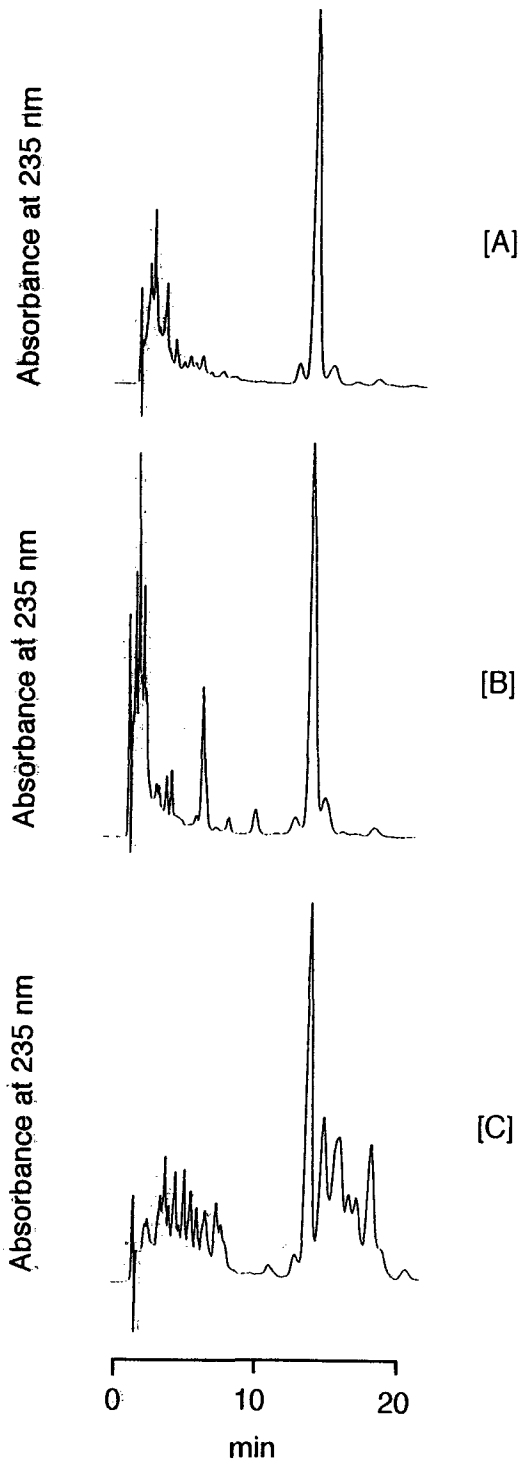


FIG. 3. HPLC chromatograms of the 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (HPETE) (A), phospholipase A_2-mediated hydrolyzed products from 1-stearoyl-2-arachidonylphosphatidylcholine after incubation with soybean lipoxygenase (B) and the isomeric mixture of HPETE from autoxidized arachidonic acid (C). Abbreviation as in Figure 1.

of α-Toc and its analogues on the ferrous ion-dependent peroxidation of PC micelles to determine the effect of these antioxidants on nonenzymatic lipid peroxidation in the phospholipid micelles. Figure 4 shows the relationship between the inhibition ratio of TBARS accumulation and various concentrations of α-Toc or its analogues. IC_{50} of α-Toc, PMC and Trolox C were 0.9, 0.3 and 7.4 μM, respectively. Inhibition ratio of α-Toc, PMC and Trolox C were calculated from the following equation:

$$\text{inhibition ratio (\%)} = ([B] - [A])/[B] \times 100 \quad [2]$$

where [*A*] and [*B*] are TBARS value after 36 h incubation in the presence of either α-Toc or α-Toc analogues (PMC and Trolox C) and that in the absence of them, respectively. Thus, the activity of these antioxidants during ferrous ion-dependent peroxidation of PC micelles was decreased in the order of PMC > α-Toc > Trolox C.

Peroxyl radical-scavenging activity of α-Toc and its analogues in solution. The peroxyl radical-scavenging activities of α-Toc and its analogues in solution were measured at their different concentrations. The ratio of the rate constant for the inhibition of oxidation during the induction period (k_{inh}) to the rate constant for chain propagation (k_p) can be determined by the following equation (27):

$$k_{inh}/k_p = [LH]/R_{inh} \cdot t_{inh} \quad [3]$$

in which [*LH*] signifies the concentration of methyl linoleate. Figure 5 shows a plot of k_{inh}/k_p vs. concentrations of antioxidants ranging from 41.7 to 166.7 μM. The value of k_{inh}/k_p for each antioxidant decreased with the increase of concentration. However, the values of k_{inh}/k_p for α-Toc and PMC were not significantly different from each other (1500–2500) at each concentration. On the other hand, the values of k_{inh}/k_p for Trolox C were slightly lower (1000–2000) than those of α-Toc and PMC. Stoichiometric numbers of radicals (*n*) trapped by PMC and Trolox C, a function of t_{inh}, were also obtained, indicating that α-Toc traps two chain-propagating lipid peroxyl radicals. The *n* values of PMC and Trolox C were calculated at different concentrations to be 1.8–2.1 and 1.9–2.3, respectively.

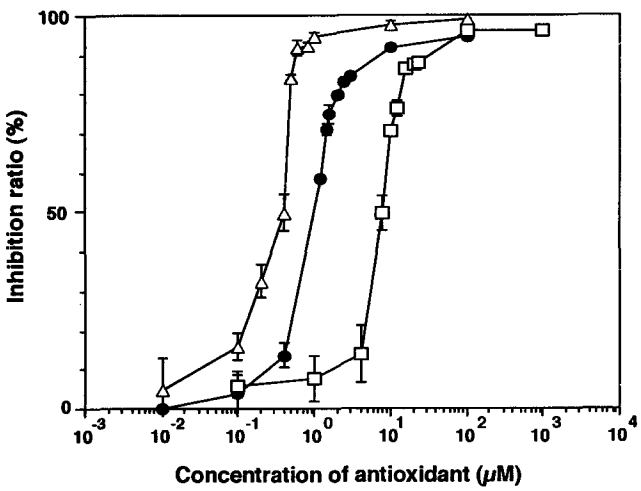


FIG. 4. The relationship between the inhibition by α-Toc (●), PMC (△) and Trolox C (□) on ferrous ion ascorbate-dependent peroxidation of PC micelles and their concentrations. Abbreviations as in Figures 1 and 2.

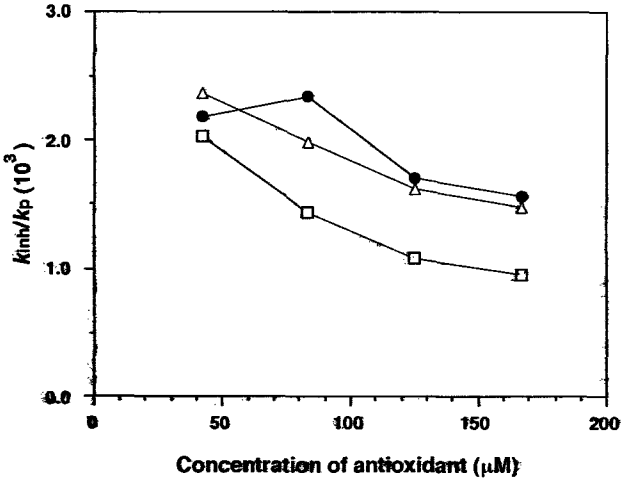


FIG. 5. The concentration-dependent rate constant for the inhibition of oxidation during the induction period and rate constant for chain propagation (k_{inh}/k_p) values of α-Toc and α-Toc analogues (PMC and Trolox C) on 2,2'-azobis-(2,4-dimethylvaleronitrile)-initiated oxidation of methyl linoleate in solution. α-Toc (●), PMC (△), Trolox C (□). Abbreviations as in Figures 1 and 2.

DISCUSSION

It has been suggested that lipoxygenase from plant and animal sources can oxidize directly fatty acids esterified in phospholipids (3–5,10,11,28–30). We selected a reaction system consisting of soybean lipoxygenase and pig liver PC in bile salt micelles to investigate the inhibitory effect of α-Toc and its analogues on the reaction of lipoxygenase with phospholipids. This simplified system will assist in studying the effect of α-Toc on lipoxygenase reaction in phospholipid bilayers.

Although the Sigma enzyme preparation was possibly a mixture of isozymes, L-1 probably predominates because it is the most stable of the isozymes. Because the 15-position of arachidonic acid moiety was exclusively oxidized from SA–PC, this demonstrates that isozyme L-1 is likely to be responsible for the hydroperoxidation because of its known specificity (21). It has been reported that α-Toc serves as an effective inhibitor of the oxidation of free fatty acids by purified lipoxygenase from plant or animal sources (31–33). However, this study is the first to report that vitamin E inhibits lipoxygenase-dependent hydroperoxidation of phospholipids as far as we know. In addition, the effectiveness of α-Toc as lipoxygenase inhibitor for phospholipids has not been compared to that of PMC or Trolox C, although Panganamala *et al.* (34) have shown that α-Toc inhibits the lipoxygenase reaction more efficiently than Trolox C in the oxidation of free fatty acids in ethanol/water solution.

Our results clearly show that PMC, an α-Toc analogue possessing a methyl group instead of phytyl side chain, exerts an inhibitory effect more efficiently than α-Toc. The efficacy of the inhibition by PMC (IC_{50}) was nearly the same as that of a synthetic antioxidant, BHA, and a known lipoxygenase inhibitor from natural origin, quercetin (35,36). Trolox C, another α-Toc analogue possessing a carboxyl group instead of phytyl side chain, exerted no inhibitory effect even at the same concentration as that of substrate PC. Interestingly, β-carotene did not act as lipoxygenase inhibitor in this reaction system although Lomniski *et al.* (37) pointed out that β-carotene inhibits lipoxygenase activity in the oxidation of free fatty acids.

In general, the inhibition of lipoxygenase reaction seems to be derived from inactivation of the active site of the enzyme or scavenging of free radicals at the active site. Niki *et al.* (13) concluded that the phytyl side chain does not affect the peroxyl radical-scavenging activity by showing that the rate constant for the inhibition (k_{inh}) by PMC was the same as that by α-Toc in the radical chain reaction of unsaturated lipids in solution. We revealed that the k_{inh}/k_p was nearly the same for α-Toc and PMC at different concentrations. The ratios obtained from Trolox C were also comparable to those obtained from α-Toc and PMC. Stoichiometric numbers of radicals (n) trapped by PMC and Trolox C were also similar to that trapped by α-Toc at different concentrations. Therefore, it is unlikely that their inherent radical-scavenging activities are the determinant for the effectiveness of inhibition on the lipoxygenase reaction of PC micelles. Thus, the localization of each compound in heterogeneous environment of the reaction system definitely affects the inhibitory effect. It seems that the localization of α-Toc, PMC and Trolox C in micelles is different from one another depending on the structure at their 2-positions. Fukuzawa *et al.* (12) and Niki *et al.* (13) have suggested that PMC has a higher motility than α-Toc in PC liposomes. It is also likely that PMC can move more freely than α-Toc in the PC micelles resulting in the superiority of PMC in inhibiting the lipoxygenase reaction. On the other hand, our results demonstrated that Trolox C has no effect on the lipoxygenase reaction. Barclay and Vinqvist (38) indicated that Trolox C is negatively charged at neutral pH because the pKa of its carboxyl group was 3.89. Therefore, negatively charged Trolox C would not enter the negatively charged bile salt micelles because of the electrostatic repulsion, and little effect of Trolox C was observed on the lipoxygenase reaction.

Once lipid hydroperoxides are formed by an enzymatic reaction, nonenzymatic chain oxidation may also occur through the generation of alkoxyl and peroxyl radicals from preformed hydroperoxides by the reaction with contaminant metal ions. It was observed that the antioxidant activity of PMC on the oxidation of PC micelles induced by ferrous ion in the presence of trace amount of PC–OOH was also superior to that of α-Toc or Trolox C. It is therefore apparent that PMC is more effective than α-Toc or Trolox C in the prevention of lipoxygenase-induced phospholipid peroxidation. Lipoxygenase-induced phospholipid peroxidation has attracted much attention in relation to the formation of oxidized low-density lipoprotein in the process of atherosclerosis (2,3) and the destruction of reticulocyte membrane structures in their maturing process (39,40). This study implies that PMC is a potent antioxidant of enzymatic peroxidation of phospholipids, although the micellar system in this experiment does not reflect directly the event of lipid peroxidation occurring *in vivo*.

REFERENCES

1. Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radical in Biology and Medicine,* 2nd edn., Clarendon Press, Oxford, pp. 299–365, 417–508.
2. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witztum, J.L. (1989) *New Engl. J. Med. 320,* 915–924.
3. Belkner, J., Wiesner, R., Rathman, J., Barnett, J., Sigal, E., and Kühn, H. (1993) *Eur. J. Biochem. 213,* 251–261.
4. Kühn, H., Belkner, J., Wiesner, R., and Brash, A.R. (1990) *J. Biol. Chem. 265,* 18351–18361.
5. Takahashi, Y., Glasgow, W.C., Suzuki, H., Taketani, Y., Yamamoto, S., Anton, M., Kühn, H., and Brash, A.R. (1993) *Eur. J. Biochem. 218,* 165–171.
6. Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radical in Biology and Medicine,* 2nd edn., Clarendon Press, Oxford, pp. 86–298.
7. Packer, L. (1992) in *Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications* (Ong, A.S.H., and Packer, L., eds.) pp. 1–16, Birkhauser Verlag, Basel.
8. Gwebu, E.T., Trewyn, R.W., Cornwell, D.G., and Panganamala, R.V. (1980) *Res. Commun. Chem. Pathol. Pharmacol. 28,* 361–376.
9. Panganamala, R.V., and Cornwell, D.G. (1982) *Ann. NY Acad. Sci. 393,* 376–391.
10. Eskola, J., and Laakso, S. (1983) *Biochim. Biophys. Acta 751,* 305–311.
11. Brash, A.R., Ingram, C.D., and Harris, T.M. (1987) *Biochemistry 26,* 5465–5471.
12. Fukuzawa, K., Chida, H., Tokumura, A., and Tsukatani, H. (1981) *Arch. Biochem. Biophys. 206,* 173–180.
13. Niki, E., Kawakami, A., Saito, M., Yamamoto, Y., Tsuchiya, J., and Kamiya, Y. (1985) *J. Biol. Chem. 260,* 2191–2196.
14. Niki, E., Takahashi, M., and Komuro, E. (1986) *Chem. Lett.* 1573–1576.
15. Takahashi, M., Tsuchiya, J., and Niki, E. (1989) *J. Am. Chem. Soc. 111,* 6350–6353.
16. Terao, J., Asano, I., and Matsushita, S. (1985) *Lipids 20,* 312–317.
17. Terao, J., and Matsushita, S. (1981) *Agric. Biol. Chem. 45,* 587–593.
18. Terao, J., Shibata, S.S., Yamada, K., and Matsushita, S. (1988) in *Medical, Biochemical and Chemical Aspects of Free Radicals* (Hayashi, O., Niki, E., Kondo, M., and Yoshikawa, T., eds.) vol. II, pp. 781–788, Elsevier Science Publishers, B.V., Amsterdam.
19. Hughes, H., Smith, C.V., Horning, E.C., and Mitchell, J.R. (1983) *Anal. Biochem. 130,* 431–436.
20. Terao, J., Shibata, S.S., and Matsushita, S. (1988) *Anal. Biochem. 169,* 415–423.
21. Funk, M.O., Isaac, R., and Porter, N.A. (1976) *Lipids 11,* 113–117.
22. Uchiyama, M., and Mihara, M. (1978) *Anal. Biochem. 86,* 271–278.
23. Terao, J., Karasawa, H., Arai, H., Nagao, A., Suzuki, T., and Takama, K. (1993) *Biosci. Biotechnol. Biochem. 57,* 1204–1205.
24. Terao, J., and Matsushita, S. (1986) *Lipids 21,* 255–260.
25. Burton, G.W., and Ingold, K.U. (1981) *J. Am. Chem. Soc. 103,* 6472–6477.
26. Weenen, H., and Porter, N.A. (1982) *J. Am. Chem. Soc. 104,* 5216–5221.
27. Niki, E., Saito, T., Kawakami, A., and Kamiya, Y. (1984) *J. Biol. Chem. 259,* 4177–4182.
28. Jung, G., Yang, D-C., and Nakao, A. (1985) *Biochem. Biophys. Res. Commun. 130,* 559–566.
29. Murray, J.J., and Brash, A.R. (1988) *Arch. Biochem. Biophys. 265,* 514–523.
30. Schewe, T., Halangk, W., Hiebsch, Ch., and Rapoport, S.M. (1975) *FEBS Lett. 60,* 149–152.
31. Grossman, S., and Waksman, E.G. (1984) *Int. J. Biochem. 16,* 281–289.
32. Reddanna, P., Rao, M.K., and Reddy, C.C. (1985) *FEBS Lett. 193,* 39–43.
33. Bakalova, R.A., Nekrasov, A.S., Lankin, V.Z., Kagan, V.E., Stoichev, T.S., and Evstigneeva, R.P. (1988) *Dokl. Akad. Nauk. SSSR 299,* 1008–1011.
34. Panganamala, R.V., Miller, J.S., Gwebu, E.T., Sharma, H.M., and Cornwell, D.G. (1977) *Prostaglandins 14,* 261–271.
35. Baumann, J., Bruchhausen, F.V., and Wurm, G. (1980) *Prostaglandins 20,* 627–639.
36. Takahama, U. (1985) *Phytochemistry 24,* 1443–1446.
37. Lomniski, L., Bar-Natan, R., Sklan, D., and Grossman, S. (1993) *Biochim. Biophys. Acta 1167,* 331–338.
38. Barclay, L.R.C., and Vinqvist, M.R. (1994) *Free Radic. Biol. Med. 16,* 779–788.
39. Rapoport, S.M., and Schewe, T. (1986) *Biochim. Biophys. Acta 864,* 471–495.
40. Kühn, H., Belkner, J., and Wiesner, R. (1990) *Eur. J. Biochem. 191,* 221–227.

[Received August 1, 1994, and in revised form December 8, 1994; Revision accepted December 23, 1994]

Protective Effect of Estrogens and Catecholestrogens Against Peroxidative Membrane Damage *in vitro*

Mercedes Lacort*, Ana M. Leal, Mariana Liza, César Martín, Rosa Martínez and M. Begoña Ruiz-Larrea

Departamento de Fisiología, Facultad de Medicina, Universidad del Pais Vasco, Bilbao, Spain

ABSTRACT: The antioxidant effects of natural estrogens (estrone, E_1; 17β-estradiol), synthetic estrogens (17α-ethynylestradiol, EE_2; mestranol, MES; diethylstilbestrol, DES) and catecholestrogens (2-hydroxyestradiol; 4-hydroxyestradiol, 4-OHE_2) on lipid peroxidation induced by different means in rat liver microsomes were investigated. The extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances. Prooxidants included Fe^{3+}/ADP/reduced NADPH, Fe^{2+}/ascorbate, *tert*-butyl hydroperoxide (*t*-BOOH) and 2,2′-azo*bis*(2-amidinopropane) (AAPH). Estrogens and catecholestrogens decreased lipid peroxidation in all four systems tested. In the iron/ascorbate model it was shown that (i) 4-OHE_2 and DES had analogous patterns of inhibition, irrespective of the presence of NADPH or the functional integrity of the microsomes, and (ii) the antioxidant activities of E_1, EE_2 and MES were dependent on the assay conditions with the activity being markedly higher when estrogen metabolism was favored. When peroxidation was initiated by the peroxyl radical generator AAPH, the inhibitory effects observed were least pronounced. Our data also showed that, in each of the systems, all inhibitors displayed the same order of inhibitory potency with DES and catecholestrogens being the most potent antioxidants under all experimental conditions used. The present results confirm earlier findings and point toward a link between estrogen metabolism and estrogen antioxidant activity. The data also indicate that estrogens and catecholestrogens interact with the peroxidative process at different levels with their interactions with iron or the metal-derived species being the most important modes of inhibition.
Lipids 30, 141–146 (1995).

Natural and synthetic estrogens have been reported to produce prooxidant and cytotoxic effects, which have been attributed to reactive intermediates generated during their microsomal metabolism (1–4). Estrogens are hydroxylated by a reduced NADPH cytochrome P-450 monooxygenase and mainly converted into catecholestrogens. Catecholestrogens and diethylstilbestrol (DES) are precursors of quinones that undergo reversible oxidation-reduction reactions (5), yielding semiquinones and reactive oxygen species (O_2^-, H_2O_2). These compounds in the presence of transition metals are a source of hydroxyl radicals, which are stimulators of lipid peroxidation. Various *in vivo* studies have demonstrated increased peroxidation in chicken erythrocytes (6) and hamster kidney (7) after estrogen treatments. Yet, other studies have shown antioxidant actions of estrogens against peroxidation induced by either ultraviolet irradiation and various prooxidant systems (8–11). We earlier found that the addition of 17β-estradiol (E_2) to fresh hepatocyte incubations protected the cells against spontaneous peroxidation (12). More recently, we have shown that E_2 and 2-hydroxyestradiol (2-OHE_2) inhibit iron-stimulated peroxidation in microsomal lipids (13). We found that E_2 was only a moderately efficient inhibitor, while inhibition increased markedly when the estrogen was incubated with microsomes (13).

The present experiments were aimed at further investigating the antioxidant action of various natural and synthetic estrogens, and catecholestrogens, on peroxidative damage to rat liver microsomes. The involvement of their metabolic transformations on these effects was also investigated. Peroxidation was induced by different types of prooxidants in order to gain a more precise view of the mechanism of inhibition. Malondialdehyde (MDA) formation was measured to monitor the extent of lipid peroxidation. The antioxidant potency of each compound with all the initiator systems was also determined by calculating the half inhibitory concentration (IC_{50}) values from the respective concentration-dependent curves (Scheme 1).

MATERIALS AND METHODS

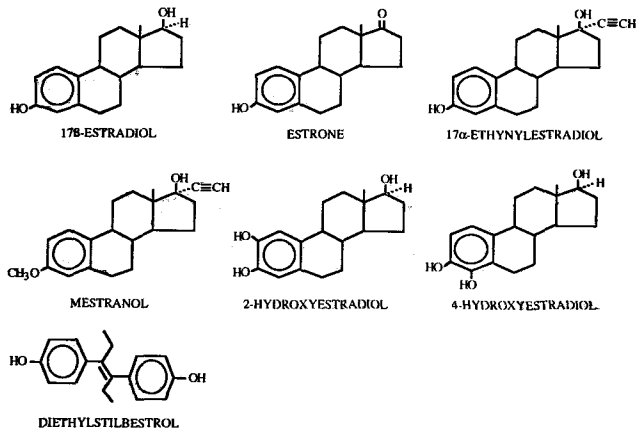
Chemicals. DES, E_2, estrone (E_1), 17α-ethynylestradiol (EE_2), 2-OHE_2, 4-hydroxyestradiol (4-OHE_2), mestranol (MES) and *tert*-butyl hydroperoxide (*t*-BOOH) (70% aqueous solution) were from Sigma (St. Louis, MO), and 2,2′-azo*bis*(2-amidinopropane) (AAPH) was from Polysciences Inc. (Warrington, PA). All other chemicals were of the highest grade available.

Preparation of microsomal fractions. Liver microsomes were isolated from male Sprague-Dawley rats weighing about

*To whom correspondence should be addressed at Departamento de Fisiología, Facultad de Medicina, Universidad del Pais Vasco, P.O. Box 699, Bilbao, Spain.

Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane); DES, diethylstilbestrol; DMSO, dimethylsulfoxide; E_1, estrone; E_2, 17β-estradiol; EE_2, 17α-ethynylestradiol; IC_{50}, half inhibitory concentration; MDA, malondialdehyde; MES, mestranol; 2-OHE_2, 2-hydroxyestradiol; 4-OHE_2, 4-hydroxyestradiol; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; *t*-BOOH, *tert*-butyl hydroperoxide.

Copyright © 1995 by AOCS Press



SCHEME 1

180 g. Rats were allowed food and water *ad libitum*. The animals were anesthetized, and the livers were perfused with saline and homogenized in 250 mM sucrose. Microsomes were prepared as previously described (14), washed once in 150 mM Tris/HCl buffer, pH 8, and suspended in 150 mM Tris/HCl buffer, pH 7.4. Denaturation of microsomes was accomplished by heat treatment (30 min at 80°C). Microsomal protein concentrations were determined by the method of Bradford (15) using bovine albumin as standard.

Incubation of microsomes. Microsomes (0.3–0.6 mg of protein) were resuspended in Tris/HCl buffer, pH 7.4, and preincubated for 10 min with the agents (10 μM) dissolved in 1% dimethylsulfoxide (DMSO) in a final volume of 1 mL. Control experiments were done using the same amount of the organic solvent. Incubations were carried out at 37°C in a shaker bath under air. At zero time, lipid peroxidation was initiated using one of the following systems: (i) Fe/ADP/NADPH: 2 μM $FeCl_3$ and 200 μM ADP (previously complexed) and 200 μM NADPH as cofactor; (ii) Fe/ascorbate: 25 μM $FeSO_4$, 100 μM ascorbate and 10 mM KH_2PO_4; in some experiments 200 μM NADPH was also added together with the estrogens; (iii) *t*-BOOH (1.5 mM) and (iv) the water-soluble azo derivative AAPH (25 mM). All additions are expressed as final concentrations. Incubations were stopped at selected time points by adding 5% trichloroacetic acid (TCA) and then centrifuged to pellet the protein.

Lipid peroxidation assay. Lipid peroxidation was measured by monitoring thiobarbituric acid reactive substances (TBARS) formation according to Fee and Teitelbaum (16). A 350-μL portion of the supernatant was added to 700 μL of a thiobarbituric acid (TBA) solution in 5% TCA, and the mixture was heated at 100°C for 15 min. After cooling, the absorbance of the sample was measured at 535 nm. Microsomal lipid peroxidation was expressed as nanomoles of MDA per milligram of protein. MDA values were calculated using a molar extinction coefficient of 156 $mM^{-1}cm^{-1}$ at 535 nm. IC_{50} values were obtained from the concentration-response curves and converted to micromolar concentrations by using a GraphPAD programme (Institut for Scientific Information, San Diego, CA).

RESULTS

We have investigated the effects of various natural and synthetic estrogens and catecholestrogens on microsomal lipid peroxidation. TBARS formation was determined in both iron-dependent and iron-independent systems. In each case, optimal reaction conditions had previously been established. DMSO itself had no effect on the peroxidative processes. None of the steroids interfered with the measurement of the TBA assay.

Figure 1 shows the time-course of TBARS formation in the Fe/ADP/NADPH system in microsomes incubated with or without the estrogens. 4-OHE_2, EE_2 and DES proved to be strong antioxidants at supraphysiologic concentrations. E_1, with a keto group at C-17, had a lower antioxidant activity. Substitution of the C-3 hydroxyl group of EE_2 by a methoxyl group (changing EE_2 to MES) greatly reduced its ability to inhibit TBARS formation. Other steroids tested (testosterone, progesterone and cholesterol) had no effects on lipid peroxidation (data not shown).

When microsomes were incubated with Fe/ascorbate, DES and 4-OHE_2 markedly suppressed MDA formation (Fig. 2a). EE_2 provoked an intermediate inhibition, whereas little or no inhibitory effects were observed in the presence of E_1 and MES, respectively. When NADPH was included during the

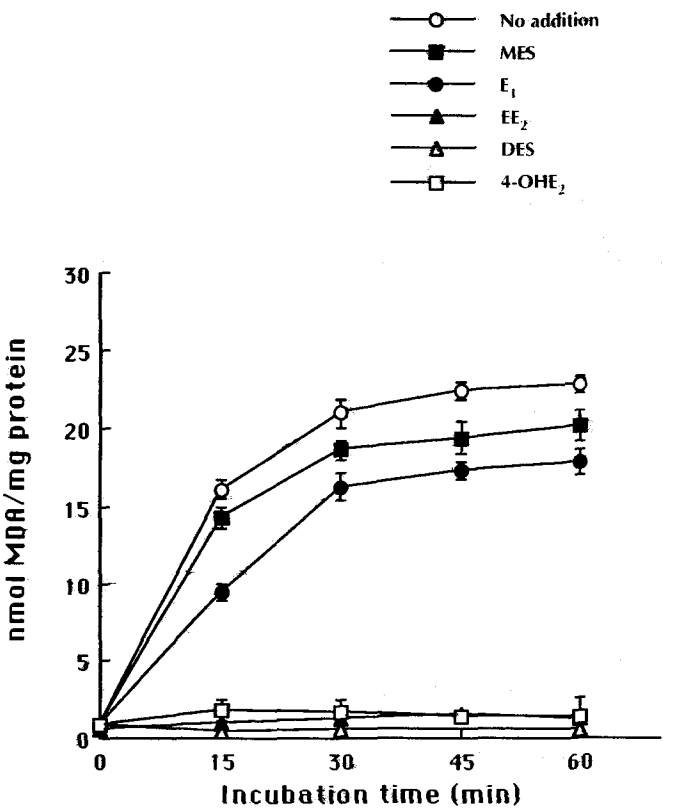


FIG. 1. Antioxidant effects of estrogens (10 μM) on microsomal lipid peroxidation induced by Fe^{3+}/ADP/NADPH. Values are the means ± SE (n = 3). MDA, malondialdehyde; MES, mestranol; E_1, estrone; EE_2, 17α-ethynylestradiol; DES, diethylstilbestrol; 4-OHE_2, 4-hydroxyestradiol.

preincubation period, thus favoring the metabolic transformation of the estrogens, all the molecules completely inhibited lipid peroxidation (Fig. 2b). Additional experiments were carried out with heat-denatured microsomes (Fig. 3); under these conditions, the patterns of inhibition of estrogens and 4-OHE_2 were similar to those obtained with functional microsomes without NADPH (Fig. 2a).

We also evaluated the kinetics of MDA formation stimulated by 1.5 mM *t*-BOOH. In comparison to iron-containing systems, the capacity of the organic hydroperoxide to promote lipid peroxidation was markedly (about 5-fold) lower (Fig. 4). Catecholestrogens and DES were the most efficient compounds, whereas MES was the least active inhibitor.

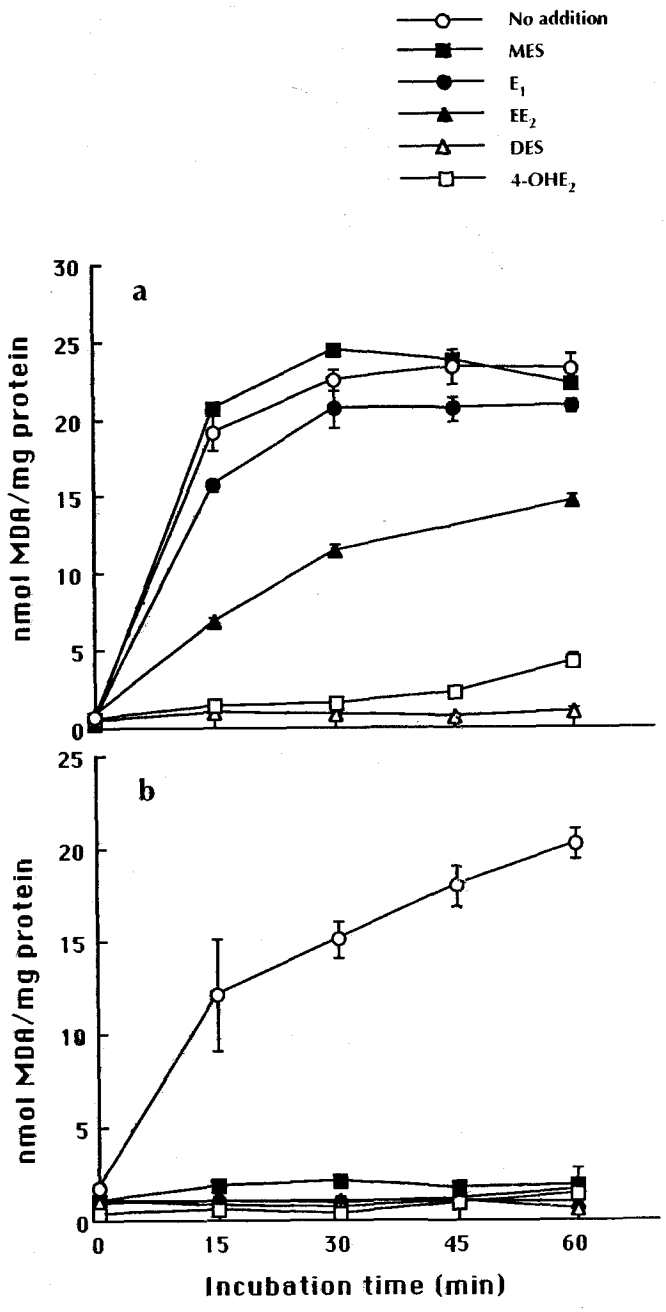


FIG. 2. Effects of estrogens on Fe^{2+}/ascorbate induced lipid peroxidation in microsomes incubated in the absence (a) or presence (b) of NADPH. Values are the means ± SE (n = 3). See Figure 1 for other abbreviations.

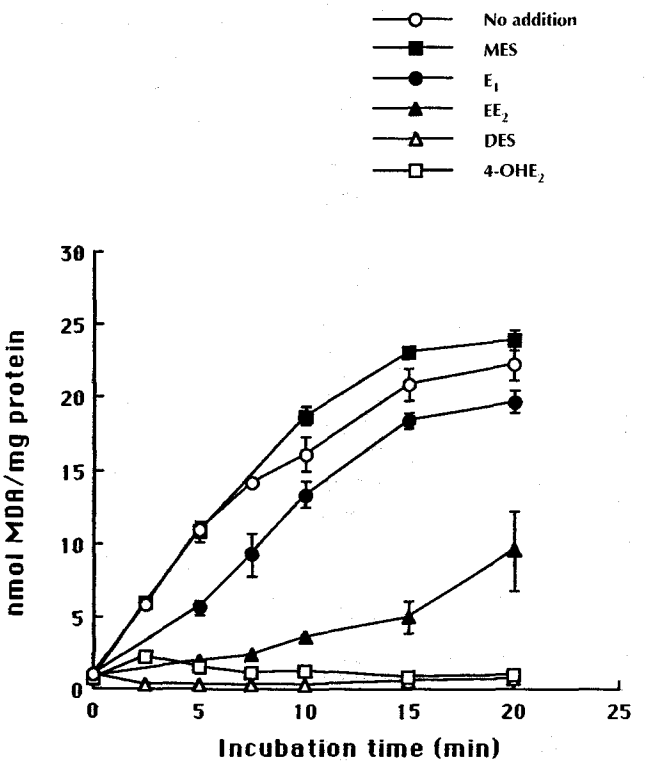


FIG. 3. Effects of estrogens on Fe^{2+}/ascorbate induced lipid peroxidation in denatured microsomes. Values are the means ± SE (n = 3). See Figure 1 for abbreviations.

Lipid peroxidation induced by the water-soluble azo compound AAPH (25 mM) was also investigated. In this model, some lipoperoxidation similar to that found with *t*-BOOH was observed in controls. The 2-OHE_2 exerted complete protection, and the other agents showed different degrees of inhibition, steroidal estrogens being the least efficient, while DES and 4-OHE_2 gave intermediate protection (Fig. 5).

In studies with denatured microsomes, only DES and catecholestrogens decreased the rates of lipid peroxidation initiated by both *t*-BOOH and AAPH, although the values of TBARS production in control incubates (without additions) were extremely low (data not shown).

We have quantified the antioxidant potential of estrogens and catecholestrogens by determining the concentration of these compounds which inhibited control MDA production by 50% (IC_{50}). Figure 6 shows curves that are typical for the agents assayed in the Fe/ADP/NADPH system. Half maximal inhibition values were calculated from these curves (or similar ones), and the results obtained are summarized in Table 1. As can be seen, the efficiencies of the compounds tested against microsomal lipid peroxidation were dependent on the initiator system, generally in the following order: Fe/ADP/NADPH > Fe/ascorbate = *t*-BOOH > AAPH. All the molecules displayed a similar order of inhibitory potency in

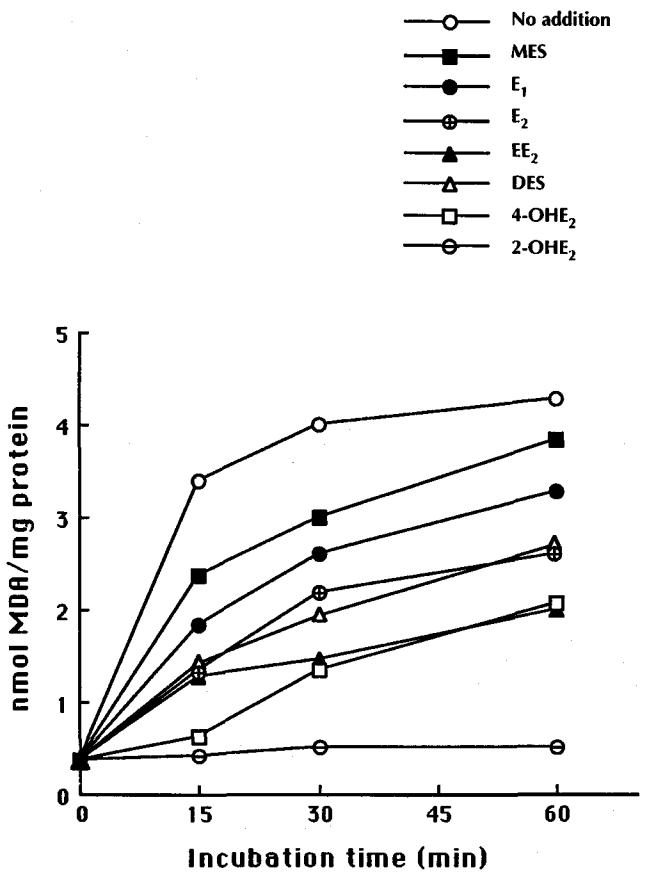


FIG. 4. Effects of estrogens and catecholestrogens on *tert*-butyl hydroperoxide induced lipid peroxidation. Values are the means from three sets of experiments with mean errors less than 5%. 2-OHE_2, 2 hydroxyestradiol; E_2, 17β-estradiol. See Figure 1 for other abbreviations.

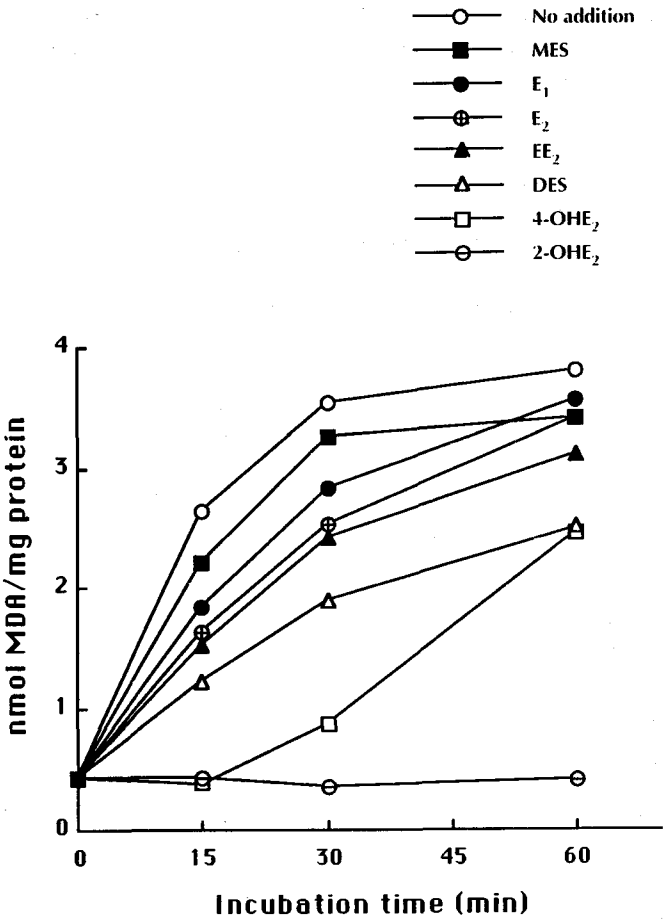


FIG. 5. Time-course of thiobarbituric acid reactive substances formation during 2,2′-azo*bis*(2-amidinopropane) (25 mM) induced lipid peroxidation in microsomes incubated with estrogens and catecholestrogens. Values are means of two experiments with mean errors less than 5%. See Figures 1 and 4 for abbreivations.

each of the systems. The catecholestrogens, with IC_{50} between 0.11–5.80 μM, were the most effective inhibitors in our systems. E_1 was found to be the least active; 50% inhibition in the AAPH model could not be achieved (Table 1).

In the Fe/ascorbate system, the efficiencies of steroidal estrogens were similar to those found with *t*-BOOH (Table 1). However, when NADPH was included in the incubates, the IC_{50} values for E_1, E_2, and EE_2 decreased to 3.6 ± 0.6 (n = 6), 2.3 ± 0.5 (n = 5) and 2.5 ± 0.5 (n = 3), respectively.

DISCUSSION

The present results provide evidence that supraphysiologic concentrations of estrogens and catecholestrogens are effective inhibitors of *in vitro* microsomal lipid peroxidation under a variety of conditions. The degrees of inhibition shown by estrogens and catecholestrogens were variable and dependent on the peroxidative model. In all cases, 2-OHE_2 was the most potent inhibitor with an IC_{50} in the range of 0.11–1.21 μM.

Several conclusions can be drawn from results obtained on the Fe/ascorbate model. 4-OHE_2 and DES showed analogous antioxidant profiles; they strongly inhibited lipid peroxidation irrespective of the functional integrity of microsomes or the presence of NADPH in the incubations. The steroidal estrogens, however, behaved differently, dependent on the assay conditions. They required preincubation with NADPH and the full activity of microsomal transforming enzymes to exert substantial inhibition. This peculiarity was likewise seen in our preliminary study dealing with E_2 effects (13), while it was not seen in other studies on estrogen antioxidant actions. The enhanced effects seen with metabolically active microsomes (Fig. 2b) are surely due to the introduction of OH groups to the steroidal structures during their transformation into catechols. Phenolic compounds have antioxidant properties because of their capacity to bind with iron (17–19) and/or to reduce peroxyl or alkoxyl radicals by donating a hydrogen atom from their OH group (20,21). Compared to the phenoxyl radical, the catechol-derived radical is resonance-stabilized and, thus, shows a higher scavenging activity. The phenolic structure of E_1 could account for the slight protective action of the estrogen seen in incubates without NADPH or when denatured microsomes were used. In this regard, the lack of an effect by MES, whose 3-OH group is blocked by methylation, is of interest. The fact that EE_2 was a better antioxidant than E_2 (Table 1) can be related to the ethynyl group at C-17 in EE_2.

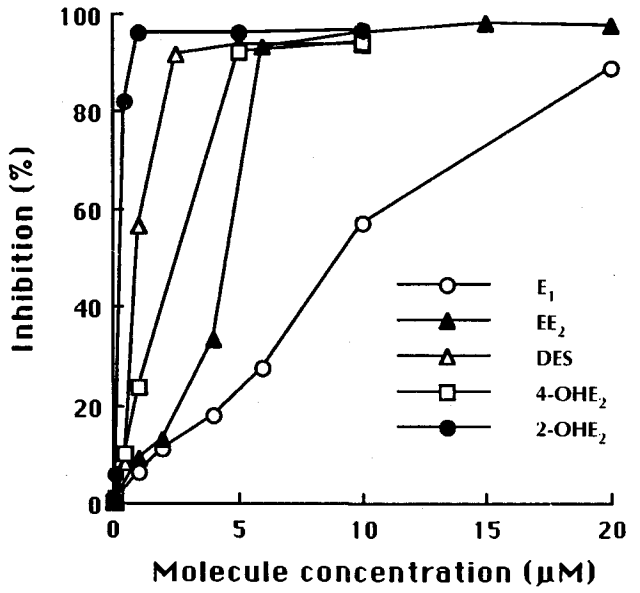


FIG. 6. Concentration-dependent inhibition of Fe^{3+}/ADP/NADPH-induced lipid peroxidation by estrogens and catecholestrogens. Microsomes were incubated for 30 min under the conditions described in the text. Results are the means of triplicate determinations. See Figures 1 and 4 for abbreviations.

Addition of Fe^{3+}/ADP/NADPH increases microsomal lipid peroxidation through the reduction of Fe^{3+} to Fe^{2+} catalyzed by NADPH cytochrome P-450 reductases. Under these conditions, estrogens may inhibit peroxidation by diverting reducing equivalents away from the iron-reduction process toward their own metabolism. The strong antioxidant action of 4-OHE_2 and DES at the concentrations used in this study makes it difficult to know whether enzymic competition is involved in the effects of these agents.

In iron-containing models, hydroxyl and perferryl radicals (22,23) and ferric/ferrous complexes (24) have been proposed as initiator species. All of them are very strong oxidants and can readily react with membrane lipids. When *t*-BOOH or AAPH were used as prooxidants, the amounts of MDA produced were much lower than those from iron-induced peroxidation. Both *t*-BOOH and AAPH generate peroxyl radicals, which are not potent oxidants (25). AAPH forms carbon-centered radicals, that rapidly react with O_2, yielding peroxyl radicals *via* an iron-independent mechanism (26,27). *t*-BOOH decomposes by heterolytic or homolytic scission, also generating peroxyl radicals, and thus accelerates propagation reactions. However, scission reactions of the hydroperoxide also produce reduced or perferryl forms of cytochrome P-450 (28) and, in this way, induce some iron-dependent peroxidation. Using *t*-BOOH as prooxidant, the antioxidant effects of the estrogens were similar to those found with Fe^{2+}/ascorbate. The inhibitory effects of these agents decreased considerably when AAPH was used as initiator. In fact, the estrogens produced the lowest inhibition in this case (Table 1).

In summary, our results corroborate preliminary reports in which a relationship between the metabolism of estrogens to catechols and their antioxidant properties was postulated. Data also show that the estrogen-protective effects on membranes depend on the chemical structure of the agent, the prooxidant used, and the availability of iron. The effects of estrogens and catecholestrogens are greater when peroxidation is initiated by complexes containing iron and less so when peroxidation involves peroxyl radicals. These results imply that there is a complex mechanism of action in the protective effects of these molecules. Estrogens and catecholestrogens seem to interact with the peroxidative process at different stages, not only by stopping free radical chain reactions at the propagation step, but also, and even more efficiently, by interacting with iron and/or the initiation species derived from the transition metal.

TABLE 1
Half Inhibitory Concentrations (IC_{50}, μM) of Estrogens and Catecholestrogens on Lipid Peroxidation Induced by Different Initiators[a]

Compound	IC_{50} (μM)			
	Fe^{2+}/asc	Fe^{3+}/ADP/NADPH	*t*-BOOH	AAPH
E_1	54.6 ± 2.9	10.8 ± 0.8	49.5 ± 2.9	NR[b]
E_2	19.3 ± 2.9	4.3 ± 0.2	18.7 ± 3.8	32.2 ± 5.7
EE_2	11.8 ± 0.5	4.2 ± 0.5	9.3 ± 1.0	28.4 ± 2.9
2-OHE_2	0.11 ± 0.03	0.34 ± 0.02	0.91 ± 0.14	1.21 ± 0.01
4-OHE_2	3.6 ± 0.5	2.2 ± 0.6	5.8 ± 0.9	ND[c]
DES	5.1 ± 0.9	1.4 ± 0.2	11.9 ± 1.9	13.7 ± 2.1

[a]Microsomes were incubated for 30 min with reaction mixtures containing lipid peroxidation inducers, as described in the text. Values are means ± SE of 3–7 experiments with different batches of microsomes. *t*-BOOH, *tert*-butyl hydroperoxide; AAPH, 2,2′-azo*bis*(2-amidinopropane); E_1, estrone; E_2, 17β estradiol; 17α-ethynylestradiol; 2-OHE_2; 2-hydroxyestradiol-4-OHE_2, 4-hydroxyestradiol.
[b]NR, not reached, i.e., inhibition does not reach 50%.
[c]ND, not done.

ACKNOWLEDGMENTS

This work was supported by Scientific Research Grant UPV 081.327-EA101/92. A.M.L. has been awarded a fellowship from the Spanish Education Ministry.

REFERENCES

1. Haaf, H., Li, S.A., and Li, J.J. (1987) *Carcinogenesis 8*, 209–215.
2. Lamartiniere, C.A., and Pardo, G.A. (1988) *J. Biochem. Toxicol. 3*, 87–103.
3. Morgan, P., Maggs, J.L., Bulman-Page, P.C., Hussain, F., and Park, B.K. (1992) *Biochem. Pharmacol. 43*, 985–994.
4. Lax, E.R. (1987) *J. Steroid Biochem. 27*, 1119–1128.
5. Liehr, J.G., and Roy, D. (1990) *Free Rad. Biol. Med. 8*, 415–423.
6. Cho, B.H., Smith, T.L., Park, J.R., and Kummerow, F.A. (1988) *Lipids 23*, 853–856.
7. Roy, D., and Liehr, J.G. (1989) *Cancer Res. 49*, 1475–1480.
8. Yagi, K., and Komura, S. (1986) *Biochem. Int. 13*, 1051–1055.
9. Sugioka, K., Shimosegawa, Y., and Nakano, M. (1987) *FEBS Lett. 210*, 37–39.
10. Nakano, M., Sugioka, K., Naito, I., Takekoshi, S., and Niki, I. (1987) *Biochem. Biophys. Res. Commun. 142*, 919–924.

11. Mooradian, A.D. (1993) J. *Steroid Biochem. Molec. Biol. 45*, 509–511.
12. Ruiz-Larrea, M.B., Garrido, M.J., and Lacort, M. (1993) *J. Biochem 113*, 563–567.
13. Ruiz-Larrea, M.B., Leal, A.M., Liza, M., Lacort, M., and de Groot, H. (1994) *Steroids 59*, 383–388.
14. Martinez, M.J., Ruiz, J.I., Lacort, M., and Ochoa, B. (1991) *Biochim. Biophys. Acta 1085*, 106–111.
15. Bradford, M.N. (1976) *Anal. Biochem. 72*, 248–254.
16. Fee, J.A., and Teitelbaum, H.D. (1972) *Biochem. Biophys. Res. Commun. 49*, 150–158.
17. Afanasév, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., and Potapovitch, A.I. (1988) *Biochem. Pharmacol. 38*, 1763–1769.
18. Pulla Reddy, A.Ch., and Lokesh, B.R. (1992) *Mol. Cell. Biochem. 111*, 117–124.
19. Weglicki, W.B., and Mak, I.T. (1992) *Mol. Cell. Biochem 118*, 105–111.
20. Liu, G., Zhang, T., Wang, B., and Wang, Y. (1992) *Biochem. Pharmacol. 43*, 147–152.
21. Halliwell, B., and Gutteridge, J.M.C. (eds.) (1989) *Free Radicals in Biology and Medicine*, 2nd edn., pp. 188–276, Clarendon Press, Oxford.
22. Puntarulo, S., and Cederbaum, A.I. (1988) *Arch. Biochem. Biophys. 264*, 482–491.
23. Samokyszyn, V.M., Miller, D.M., Reif, D.W., and Aust, S.D. (1989) in *Membrane Lipid Oxidation* (Vigo-Pelfrey, C., ed.) Vol. I, pp. 101–129, CRC Press, Boca Raton.
24. Minotti, G., and Aust, S.D. (1987) *Chem. Phys. Lipids 44*, 191–208.
25. Scheschonka, A., Murphy, M.E., and Sies, H. (1990) *Chem. Biol. Interactions 74*, 233–252.
26. Yamamoto, Y., Haga, S., Niki, E., and Kamiya, Y. (1984) *Bull. Chem. Soc. Japan. 59*, 1260–1264.
27. Barclay, L.R.C., Locke, S.J., MacNeil, J.M., and VanKessel, J. (1984) *J. Am. Chem. Soc. 106*, 2479–2481.
28. Cadenas, E., and Sies, H. (1982) *Eur. J. Biochem. 124*, 349–356.

[Received March 10, 1994, and in revised form November 10, 1994; Revision accepted December 5, 1994]

Effect of Exercise on the Plasma Nonesterified Fatty Acid Composition of Dogs and Goats: Species with Different Aerobic Capacities and Diets

Grant McClelland[a,1], Georges Zwingelstein[b], C. Richard Taylor[c] and Jean-Michel Weber[a,*]

[a]Biology Department, University of Ottawa, Ottawa, Ontario, K1N 6N5 Canada, [b]Institut Michel Pacha, Université de Lyon, 83500 La Seyne sur Mer, France and [c]Concord Field Station, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT: The goals of this study were to determine: (i) whether mammals mobilize particular nonesterified fatty acids (NEFA) preferentially during locomotion, (ii) if differences in aerobic capacity or diet can affect the pattern of NEFA mobilization and (iii) which individual NEFA are most representative of total NEFA concentration changes, to use them as tracers for turnover studies. Individual NEFA were measured in trained dogs and goats (VO_2max dog/VO_2max goat = 2.2; where VO_2max = maximal oxygen consumption) during treadmill exercise at 40 and 60% VO_2max. Important interspecies differences in individual NEFA concentrations could be attributed to differences in aerobic capacity. The more aerobic species (dog) had much higher plasma NEFA concentrations for all but one NEFA (18:0), when compared with the low-aerobic species (goat). In addition, exercise caused a large increase in concentration of individual NEFA in the dogs, with the largest increases seen in 18:1 (150% above resting values) and 16:0 (60% increase), but it had no effect in goats. Therefore, the aerobic species has a much higher ability for mobilizing and transporting NEFA in plasma than its low-aerobic counterpart. Two NEFA accounted for more than half total plasma NEFA in both species, 18:1 (about 35% total NEFA) and 16:0 (20%). Calculation of variability in percent composition reveals that oleate and palmitate also closely reflect changes in total NEFA and are therefore the most appropriate tracers for *in vivo* kinetic studies in exercising mammals. Differences in diet and digestion physiology explained some differences in the plasma NEFA composition of the two species; this was reflected in the percent contributions of individual fatty acids to total NEFA: in dogs 18:1 > 16:0 > 18:2 > 18:0, while in goats 18:1 > 16:0 > 18:0 > 18:2. Also, only goats had 18:3 (6% total NEFA), a fatty acid of plant origin, while only dogs had 16:1 (7% total NEFA). Overall, however, the plasma NEFA composition of goats did not exactly reflect that of their diet due to preabsorptive modification of unsaturated fatty acids, while in dogs there was a good correlation between dietary and plasma NEFA.

Lipids 30, 147–153 (1995).

Fatty acids represent a crucial source of oxidative fuel for muscle contraction, and vertebrates are known to store enough triacylglycerol to sustain exercise for several days (1,2). Mammals adapted for prolonged locomotion can oxidize nonesterified fatty acids (NEFA) at much higher rates than sedentary, low-aerobic species (3,4), in part because of their increased capacity to mobilize NEFA from adipose tissue (5), but also because of their higher rates of circulatory NEFA transport (4). At equivalent exercise intensities (same percentage VO_2max; where VO_2max = maximal oxygen consumption), endurance-adapted mammals transport NEFA at much higher rates than do low-aerobic species, possibly because the plasma albumins of such species allow them to maintain a much higher total NEFA concentration in plasma (4). Major changes in total plasma concentration occur during and after exercise (4,6), but it is not clear whether all individual NEFA change in parallel, or if there is preferential mobilization or use of certain fatty acids (7).

Dogs and goats were chosen as representative species of endurance-adapted and sedentary mammals for this and other studies (3–5,8) because their aerobic capacities (VO_2max) differ by a factor of 2.2. In addition, these species show major differences in diet and digestive physiology (9,10). In nonruminant mammals, including dogs and humans, plasma NEFA patterns closely reflect the fatty acid composition of adipose tissue triacylglycerol (7), and the composition of stored triacylglycerol is itself closely related to dietary fatty acid patterns (10,11). In contrast, ruminant species, such as the goat, show important preabsorptive modifications of NEFA. For example, a significant portion of 18 carbon polyunsaturated NEFA are hydrogenated to stearate (9). These significant differences in energy metabolism, diet and digestion suggest that dogs and goats should differ in their plasma NEFA patterns, and that exercise should have a different effect on the two species.

Preferential mobilization and oxidation of particular NEFA also influence the choice of a proper fatty acid tracer for *in vivo* kinetics studies of lipid fluxes in mammals, and the same individual NEFA may not be an appropriate tracer for all species. NEFA turnover rate is usually measured by

[1]Present address: Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, B.C., V6T IZ4 Canada.

*To whom correspondence should be addressed at Biology Department, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, KIN 6N5 Canada.

Abbreviations: ANOVA, analysis of variance; CV, coefficient of variation; GC, gas chromatography; NEFA, nonesterified fatty acid; VO_2, rate of oxygen consumption; VO_2max, maximal rate of oxygen consumption or aerobic capacity.

Copyright © 1995 by AOCS Press

continuous infusion of a labelled fatty acid, and this technique is based on the assumption that the individual NEFA used as a tracer is representative of the total NEFA pool (12). Traditionally, palmitate and oleate have been chosen almost exclusively because they are the most abundant in human plasma (13,14). However, it is not known whether these particular NEFA can be used interchangeably in other species or if other acids would be more suitable. As a first attempt to address this issue, and because concentration and flux are usually correlated (13,15), we have quantified changes in percent composition for each plasma NEFA and calculated its coefficient of variation (CV) throughout conditions of extreme fluctuations in total concentration (i.e., exercise and recovery). Theoretically, the NEFA whose percentage composition shows the lowest CV is the best representative of the total NEFA pool. The goals of this study were, therefore, to measure the concentration of individual NEFA in the plasma of exercising dogs and goats: (i) to determine whether particular NEFA are mobilized or used preferentially during locomotion, (ii) to determine whether differences in diet or aerobic capacity can have an effect on the pattern of NEFA mobilization and (iii) to find the most appropriate NEFA tracer(s) for *in vivo* kinetic studies by establishing which NEFA best represents the total plasma NEFA pool in the two species.

MATERIALS AND METHODS

Animals, training and experimental protocols. All experimental procedures were approved by the Animal Care Committee of Harvard University (Cambridge, MA). Five months before the experiments, three African pygmy goats *(Capra hircus,* two females and one male, body mass 31.0 ± 1.3 kg) and three dogs *(Canis familiaris,* all females, 24.9 ± 1.8 kg) were started on a training schedule using a motorized treadmill at different speeds. Animals were trained at least four times weekly until their oxygen consumption (VO_2) was reproducible at each speed. VO_2 was measured with an open flow system as described by Fedak *et al.* (16). All training and experiments were performed at 18 and 29% incline for goats and dogs, respectively. To allow meaningful comparisons, exercise intensities were standardized according to maximal oxygen consumption (VO_2 max), and the two species were always measured at the same percentage of their VO_2max. The VO_2max of the individual animals used here had been measured previously (3), and each animal was specifically trained to run for 2 h at 40% VO_2max and 1 h at 60% VO_2max. In the present study, 60% VO_2max was chosen as the highest intensity because lipolysis (5) and circulatory fatty acid delivery (4) are both maximized at this work rate. Recovery was also monitored because the highest plasma fatty acid concentrations are known to occur shortly after the end of exercise in several species including dogs (4) and humans (6,17).

Goats were fed hay (NEFA composition: 25.5% 16:0, 0.4% 16:1, 3.1% 18:0, 14.2% 18:1, 27.4% 18:2 and 26.5% 18:3) and they were housed in a large outdoor paddock with shelter. The dogs were fed dry dog chow (5% fiber, 25% protein and 10% fat with the following composition: 24.5% of total NEFA as 16:0, 0.3% 16:1, 14.6% 18:0, 42.1% 18:1 and 15.5% 18:2), and they were housed in a large indoor-outdoor run. Food was withheld for 18 h before measurements, but the animals had unlimited access to water. One day before each experiment, a PE-50 catheter was placed in the aorta *via* the carotid artery as described previously (5). Blood samples (2 mL each) were drawn 15, 10 and 5 min before exercise, and at times 0, 2, 5, 10, 20, 40, 60, 80, 100 and 120 min during exercise at 40% VO_2max. At 60% VO_2max, samples were taken at times 0, 2, 5, 10, 20, 30, 40, 50 and 60 min. Additional samples were drawn 2, 5, 10, 20, 30, 40, 50 and 60 min after exercise had ended (recovery period). Samples were spun, and the plasma was separated and frozen immediately. The two exercise intensities were measured in different experiments, and two experiments on the same animal were separated by at least one week.

NEFA concentrations. Individual NEFA concentrations were measured on a Hewlett-Packard 5890 series II gas chromatograph (Mississauga, Canada) equipped with an automatic injection system (HP 7673) and flame-ionization detector. NEFA extraction and methylation were performed with the method of Tserng *et al.* (18) modified by increasing methylation time to 20 min. Preliminary thin-layer chromatography (TLC) and gas chromatography (GC) analyses using plasma and standard polyunsaturated fatty acids, including 20:4ω6, showed that 20 min were necessary for near complete methylation. One-hundred µL of plasma were methylated with 1 mL 2,2-dimethoxypropane (Sigma, St. Louis, MO) after adding 20 µL of conc. HCl. The reaction was stopped after 20 min by the addition of 10 µL pyridine, and the NEFA methyl esters were recovered by two extractions with 750 µL isooctane (BDH, Toronto, Ontario, Canada). Analyses were performed on a 30-m fused silica capillary column (DB-23; J&W Scientific, Folsom, CA) kept at 170°C for 35 min after injection, raised to 220°C at a rate of 50°C/min, and kept at that temperature up to 46 min. The injection port temperature was 210°C and the detector was kept at 250°C. NEFA standards (Supelco, Bellefonte, PA) were injected for GC calibration, and heptadecanoic acid (12 µg/100 µL, in methanol) was used as an internal standard. This internal standard was added to plasma before methylation, and recovery ranged between 95 and 98%.

Statistical analysis. Results were analyzed with one- and two-way analyses of variance (ANOVA) after percentages were arcsine transformed. In cases where the data failed tests for normality (Kolmogorov–Smirnov), a nonparametric Kruskal–Wallis one-way ANOVA on ranks was used. Bonferroni's *t*-test for multiple comparisons was used to compare mean NEFA concentrations during exercise and recovery with those at rest, and to compare the different phases in Table 1. A Dunn's multiple comparison was performed when sample sizes were unequal. Linear regressions were performed on all exercise values for each species separately to see if NEFA concentrations changed significantly over time.

TABLE 1
Fatty Acid Composition[a] (wt% total fatty acids) in Plasma of Dogs and Goats Before, During and After Exercise at Two Intensities[b]

Phase	Fatty acid (wt%)				
	Dogs: 40% VO_2max				
	16:0	16:1	18:0	18:1	18:2
a	24.0 ± 0.2[c,d]	5.5 ± 0.2	12.7 ± 2.0	35.4 ± 1.8	22.5 ± 0.4[c,d,e]
b	25.4 ± 0.5[e]	5.6 ± 0.1	14.6 ± 2.1	31.9 ± 2.0	22.5 ± 0.4[f–h]
c	23.5 ± 0.4[f]	8.4 ± 0.6	11.1 ± 2.2	38.0 ± 2.5	19.7 ± 0.8[c,g]
d	20.4 ± 0.8[c,e]	8.6 ± 0.8	8.6 ± 1.6	42.6 ± 2.1	19.8 ± 0.6[d,g]
e	19.8 ± 1.2[d,f]	5.3 ± 0.6	11.2 ± 1.1	35.3 ± 5.3	18.0 ± 0.6[e,h]
	Dogs: 60% VO_2max				
	16:0	16:1	18:0	18:1	18:2
a	22.8 ± 0.6[c,d]	5.0 ± 0.9	15.7 ± 2.4	36.7 ± 2.1[c]	19.8 ± 0.2
b	23.4 ± 0.5[e,f]	4.8 ± 1.0	19.3 ± 3.0	32.4 ± 2.0[d,e]	20.3 ± 0.3[c]
c	22.6 ± 0.2[g,h]	8.2 ± 1.3	13.1 ± 2.4	38.1 ± 0.9	18.3 ± 0.5
d	19.2 ± 0.3[d,f,g]	8.8 ± 1.6	9.8 ± 1.7	44.6 ± 0.7[c,e]	17.7 ± 0.4[c]
e	20.0 ± 0.5[c,e,h]	4.8 ± 0.7	15.8 ± 2.3	40.7 ± 1.7[d]	18.7 ± 0.5
	Goats: 40% VO_2max				
	16:0	18:0	18:1	18:2	18:3
a	21.8 ± 0.8	30.9 ± 0.4[c]	31.7 ± 1.2	9.6 ± 0.7	6.1 ± 0.4
b	25.0 ± 1.0	22.2 ± 1.5[c,d]	37.7 ± 1.9	8.6 ± 1.0	6.5 ± 0.5
c	24.9 ± 1.0	25.7 ± 1.8	35.2 ± 2.7	8.8 ± 1.6	5.3 ± 0.9
d	22.9 ± 0.5	26.5 ± 1.6	37.6 ± 2.8	8.3 ± 1.4	4.4 ± 0.6
e	22.3 ± 0.6	31.6 ± 1.6[d]	30.1 ± 2.7	10.0 ± 1.4	6.1 ± 0.8
	Goats: 60% VO_2max				
	16:0	18:0	18:1	18:2	18:3
a	17.0 ± 1.1[c,d,e]	37.6 ± 1.6[c,d,e]	26.5 ± 2.4[c,d,e]	8.1 ± 1.3	6.5 ± 0.9
b	22.3 ± 1.0[e,f]	27.1 ± 2.2[e,f]	38.1 ± 2.0[c,f,g]	7.3 ± 0.5	6.3 ± 0.2
c	21.9 ± 1.3[c]	27.3 ± 2.4[c,g]	39.0 ± 1.3[d,h,i]	7.0 ± 0.8	5.8 ± 0.4
d	22.0 ± 0.9[d]	27.3 ± 1.7[d,h]	40.1 ± 2.5[e]	6.7 ± 0.7	5.5 ± 0.8
e	17.4 ± 1.1[f]	37.7 ± 1.5[f,g,h]	28.4 ± 1.7[f,h,i]	9.6 ± 1.6	6.1 ± 0.5

[a]Values are averages of two determinations in each of three animals.
[b]See Figure 2 and Results section for definition of phases a through e. Animals were exercised at 40 and 60% VO_2max (VO_2max = maximal oxygen consumption).
[c–i]Within each column and each treatment, means for phases sharing the same superscript are significantly different from each other at $P < 0.05$.

CVs (19) were calculated separately in each species for each NEFA throughout exercise and recovery. Data for the two exercise intensities were combined to calculate these coefficients. All values presented are means and their standard errors (SEM).

RESULTS

NEFA concentrations. Plasma concentrations of palmitic, oleic and linoleic acids were always much higher in dogs than in goats before, during and after exercise at 40 and 60% VO_2max ($P < 0.001$, Fig. 1). Stearic acid concentration was not different between the two species (263 ± 6 nmoles/mL) and remained at resting levels throughout exercise at both intensities and during recovery. In dogs, with the exception of stearate, the concentration of all NEFA showed a steady increase during exercise at both intensities (linear regressions, $P < 0.01$), and an overshoot in concentration occurred after the end of work (Fig. 1). NEFA concentrations were maximal during the overshoot when they reached levels more than twice resting values shortly after exercise ended ($P < 0.05$). Dog 16:0 reached maximal concentrations of 690 ± 47 and 649 ± 78 nmol/mL shortly after exercise at 40 and 60% VO_2max, respectively. In the same species, 18:1 concentration increased throughout exercise and reached maximum values of 1495 ± 153 and 1479 ± 109 nmol/mL, 20 min after ending exercise at 40 and 60% VO_2max, respectively. Recovery overshoot values for 18:2 were 678 ± 23 nmol/mL (20 min after the 40% VO_2max run) and 614 ± 76 nmol/mL (10 min after the 60% VO_2max run) (Fig. 1). In sharp contrast to the response observed in dogs, the NEFA concentrations of goats never increased above resting values ($P > 0.05$). Palmitic acid remained at an average level of 153 ± 8 nmol/mL, oleic acid stayed at 227 ± 13 and linoleic acid at 66 ± 5 for both, 40 and 60% VO_2max runs ($P > 0.05$, Fig. 1). Within each species, the overall patterns of changes in NEFA concentration were similar at the two exercise intensities.

NEFA composition. Palmitic, stearic, oleic and linoleic acids were present in both species. Significant amounts of 16:1 were only found in dog plasma (7% of total NEFA), and 18:3 was present only in goat plasma (6%). Changes in the distribution of individual NEFA over time are presented in

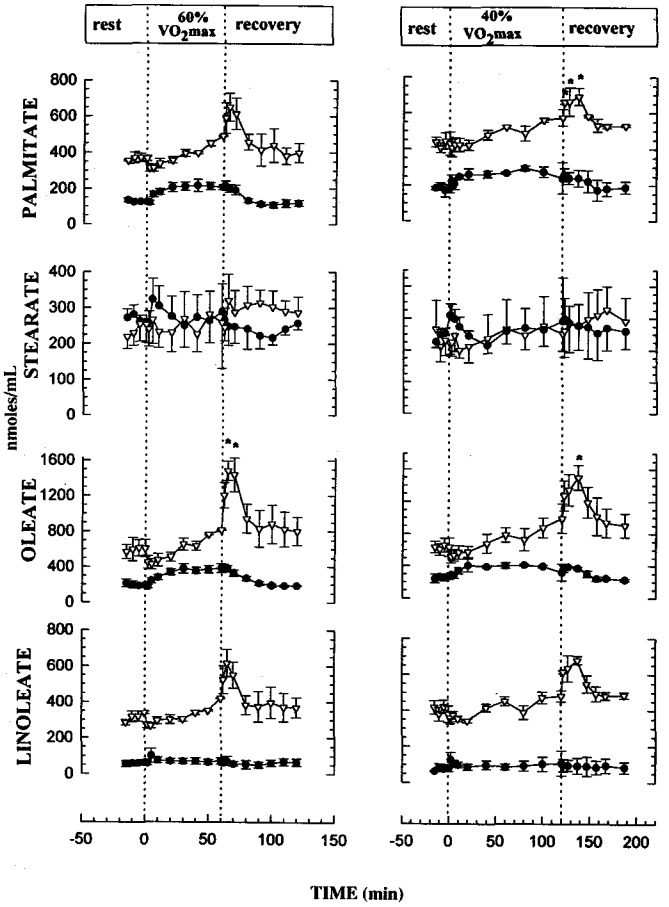


FIG. 1. Mean concentrations of individual nonesterified fatty acid in the plasma of dogs (▽, n = 3) and African pygmy goats (●, n = 3) before, during and after a 1-h run at 60% maximal oxygen consumption (VO_2max) and a 2-h run at 40% VO_2max.

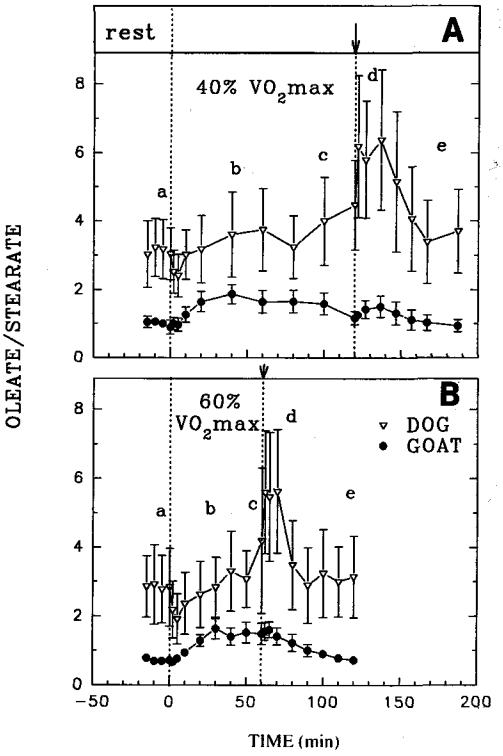


FIG. 2. Ratio of oleic to stearic acid concentration in the plasma of dogs (▽, n = 3) and goats (●, n = 3), before, during and after exercise (A) a 2-h run at 40% VO_2max and (B) a 1-h run at 60% VO_2max. Arrows indicate when exercise was terminated. See Figure 1 for abbreviation.

Table 1 as percentages of total NEFA concentration. Experimental protocols were divided in five phases to facilitate comparison: Phase a was the preexercise control period. Phase b was the onset of exercise (average value between 20 and 40 min of exercise at 40% VO_2max, and between 20 and 30 min at *60%* VO_2max). Phase c was the end of exercise (average value between 100 and 120 min at 40% VO_2max, and between 50 and 60 min at 60% VO_2max). Phase d was the beginning of recovery (127 to 137 min at 40% VO_2max and 65 to 70 min at 60% VO_2max). Finally, phase e was the end of recovery (167 to 187 min at 40% VO_2max and 110 to 120 min at 60% VO_2max). The five phases are illustrated in Figure 2, and significant differences between phases are indicated by superscripts in Table 1. Oleic acid was the most abundant NEFA, representing on average over 35% of total NEFA in both species. Similarly, the relative importance of 16:0 was the same in dogs and goats; averaged throughout exercise and recovery at both intensities, 16:0 accounted for 21% of total NEFA concentration. Together, therefore, 18:1 and 16:0 represented more than half of total NEFA in both species. At 40% VO_2max, the percentages of 18:1 and 16:0 were not different between species ($P > 0.05$). However, at 60% VO_2max, the percentages of 18:1 and 16:0 were both significantly higher in dogs than in goats ($P < 0.01$) (39 vs. 34% for 18:1, and 22 vs. 20% for 16:0). The percentage of 18:0 was almost two times higher in goats than in dogs (goats: 24 and 27% vs. dogs: 13 and 16% at 40 and 60% VO_2max, respectively). The situation was reversed for 18:2 because this NEFA accounted for 20% in dogs, but only 9% in goats.

To emphasize important interspecies differences in the effect of exercise on plasma NEFA composition, the concentration ratio of 18:1 to 18:0 was calculated for both species (Fig. 2). This ratio was always much higher in dogs than in goats ($P < 0.001$). The difference between the two species varied during and after exercise at both intensities, but it was most pronounced early in recovery. At rest, the ratio was more than 2-fold higher in dogs than in goats (over 2.5 vs. 0.9). The ratio in goats doubled during exercise before returning progressively to resting levels during the recovery period. In dogs, the ratio increased slowly during exercise before a much sharper rise in the first 20 min of recovery. High maximal values of 5.4 and 5.0 were reached after ending the runs at 40 and 60% VO_2max, respectively (Fig. 2).

CVs for individual NEFA composition. Mean CVs for all exercise and recovery values at both 40 and 60% VO_2max were calculated for the percent composition of each NEFA (Table 2). Palmitic and oleic acids had the lowest and second lowest CV in both species, respectively. The variability in composition of all the other NEFA was clearly higher than for

TABLE 2
Mean Coefficients of Variation for Individual Fatty Acid Composition (% total nonesterified fatty acid concentration) Throughout Exercise and Recovery in Trained Goats and Dogs[a]

	Goat	Dog
16:0	9.06	8.86
16:1	—[b]	22.21
18:0	13.07	19.11
18:1	12.30	10.09
18:2	16.70	16.43
18:3	15.63	—[b]

[a]Values for two exercise intensities were combined (40 and 60% maximal rate of oxygen consumption or aerobic capacity). [b]Not detected.

16:0 or 18:1, and the highest CVs were observed for 16:1 in dogs and 18:2 in goats.

DISCUSSION

The present study shows that exercise has a very different effect on the concentration of individual plasma NEFA in sedentary animals, such as goats, than in highly aerobic, endurance-adapted animals, such as dogs. It also reveals that important differences in plasma NEFA composition between these two species can be attributed to dietary NEFA and differences in digestion. Moreover, variability in percent composition for individual NEFA shows that, as in humans, changes in plasma 16:0 and 18:1 most closely reflect changes in total NEFA concentration of dogs and goats, suggesting that these two fatty acids are most appropriate as tracers for NEFA kinetic studies in mammals in general.

Except for stearic acid, plasma NEFA concentrations were much higher in dog than in goat (Fig. 1), allowing the species with high aerobic capacity (dog) to deliver this important oxidative fuel to working muscles at much higher rates than the sedentary species with low aerobic capacity (goat). The high concentrations found in dogs may be possible because this species possesses a plasma albumin with higher NEFA binding capacity than goat albumin. We have recently suggested this higher albumin binding capacity for fatty acids in dogs as an adaptation for endurance locomotion (4).

Another major difference between the two species lies in the fact that exercise causes a large increase in NEFA concentration in dogs (except for 18:0), but has no effect in goats, where it stays at resting levels throughout work and recovery (Fig. 1). Humans have a similar response to that described here for dogs: except for 18:0, their plasma NEFA concentration increases by 25% after exercising at moderate intensity for 30 min (21). These results clearly show that dogs and humans mobilize NEFA reserves during exercise, because, in these species, concentrations of individual NEFA are known to be well correlated with rates of turnover (13,15) and oxidation (15,20). Therefore, in dogs and humans, increases in concentration can be used as an index of NEFA mobilization. In contrast, the lack of change in plasma NEFA concentration of exercising goats reveals that they are able to increase NEFA flux while keeping plasma concentration constant. In goats, NEFA mobilization and oxidation are kept closely balanced throughout exercise and recovery. This observation shows that concentration and turnover of 16:0 and 18:1 are not correlated in goats because they do increase oxidation during exercise, even though lipid utilization is not stimulated as much as in dogs (4).

In dogs, 18:1 and 16:0 have the highest concentrations at rest and show the greatest relative increase during the onset of exercise. These results indicate that dogs mobilize these two NEFA preferentially compared with other NEFA, and probably at much higher rates than do goats. However, these conclusions must remain tentative because percent extraction in locomotory muscles may change with exercise and between species. For example, stearic acid does not follow the typical pattern observed for all other NEFA, and it does not appear to contribute to the overall increase in oxidative fuel use associated with exercise in mammals studied to date. It has been proposed that the fractional uptake of 18:0 may increase in exercising humans (21), suggesting that rates of release from adipose tissue and uptake by working muscles may both increase in parallel and stay exactly matched during physical work (17). This would make stearic acid flux independent of its plasma concentration, and an increase in 18:0 mobilization/oxidation would only be discernible by flux measurements. Stearic acid kinetics should be investigated thoroughly in exercising mammals to determine whether the scenario proposed above actually occurs. In goats, the absence of NEFA mobilization throughout exercise may be the consequence of high plasma glucose availability. Recent experiments on the same animals reveal that glucose concentration increases progressively during exercise in goats, but is very steady in dogs (Weber, Roberts and Taylor, unpublished results). Hyperglycemia can decrease fatty acid turnover by as much as 30% in humans (22), and glucose could therefore be an important inhibitor of lipid mobilization in exercising goats.

Dogs (Fig. 1) and humans (6,17) show a very large post-exercise increase in NEFA concentration. Fifteen min after the 40% VO_2max run, dog 16:0 and 18:1 concentrations reached maximal values 60 and 150% above resting levels, respectively. In striking contrast, no change was observed in recovering goats. The large concentration overshoots seen in dogs and humans indicate that both these species, unlike goats, probably lack rapid control mechanisms for lipolysis (5,6,17). High plasma NEFA concentrations after exercise may play a role in the regulation of fuel oxidation by inhibiting muscle glycolysis through the glucose–fatty acid cycle, and by promoting muscle glycogen resynthesis through an increase in glucose availability (17). In goats, the absence of NEFA overshoot is consistent with the fact that their lipolytic rate returns to resting levels immediately and rapidly after the end of exercise (5).

Plasma NEFA compositions of the two species investigated can be explained by differences in diet, incorporation of individual NEFA in triacylglycerols, and *in situ* modifications of NEFA in adipose tissue and liver triacylglycerol reserves. The composition of NEFA in plasma is known to reflect differen-

tial NEFA incorporation into adipose tissue triacylglycerol, and it has been reported that under conditions of enhanced lipolysis, the composition of plasma NEFA approaches that of adipose tissue (7). *In vivo* dog experiments and *in vitro* measurements on rat epididymal fat pads show that the rate of release of each individual NEFA is correlated with its relative concentration in adipose tissue (7). For nonruminants, both adipose tissue triacylglycerol and plasma NEFA reflect dietary composition (11) due to little preabsorptive modification (10). Indeed, the composition of plasma NEFA in dogs (Table 1) closely reflects that of their diet (see Materials and Methods section). In ruminant mammals, NEFA composition of adipose tissue triacylglycerol may also be influenced by postabsorptive modifications of dietary NEFA. For example, the much higher proportion of 18:1 found in goat plasma (Table 1) than in their diet may be the consequence of high activities for the enzymes fatty acid elongase and stearoyl-CoA desaturase in adipose tissue (23,24).

Stearic acid represents a greater proportion of total dietary NEFA in dogs than in goats, but the opposite is true for total plasma NEFA (Table 1). This discrepancy can be accounted for by differences in digestive physiology between ruminants and nonruminants. In ruminants, dietary unsaturated fatty acids are hydrogenated in the rumen, and little polyunsaturated NEFA reach absorption sites in the small intestine (9,25). For example, higher than expected levels of stearic acid are probably found in plasma because a large proportion of dietary 18 carbon polyunsaturated NEFA is hydrogenated to 18:0 prior to absorption (9). Given that goats and other ruminants absorb lower amounts of polyunsaturated NEFA than predicted from their diet (25), availability from digestion rather than differential rate of esterification between NEFA in adipose tissue triacylglycerol likely dictates the composition of adipose tissue and ultimately plasma (23).

Figure 2 shows that the concentration ratio of 18:0/18:1 is very different between dogs and goats, before, during and after exercise. This observation may be partly explained by interspecific differences in the activities of enzymes responsible for NEFA incorporation in adipose tissue triacylglycerol; lower incorporation of particular NEFA may result in their lower contribution to total plasma NEFA. Except for stearic acid, rates of incorporation in adipose tissue increase linearly with plasma concentration (23). In experiments by Lin *et al.* (23), 18:0 had the lowest rate of esterification in bovine adipose tissue while 16:0 had the highest. Incorporation of 18:0 in adipose tissue triacylglycerol may differ between dogs and goats due to discrepancies in the activities of fatty acyl-CoA synthetase. Low incorporation of stearic acid into bovine adipose tissue is thought to be caused by fatty acyl-CoA synthetase's low affinity for 18:0 (23). As in dogs, 18:0 does not contribute to the increase in total plasma NEFA of humans, and the 18:1/18:0 ratio increases from 3.3 at rest (compared to 2.7 in dogs) to a maximum of 4.5 (5.4 in dogs) after 50 min of exercise (21).

Additional differences between dogs and goats may be more directly attributed to differences in diet, and the composition of plasma NEFA found here in goats is comparable with values reported previously (25). Only goat plasma contained 18:3, a NEFA of plant origin especially abundant in grass lipids (25,26), and represented a relatively large proportion of total FA in the goat diet of our study (see Materials and Methods section). Palmitoleic acid was only found in dog plasma, although this NEFA has also been reported to be present in small amounts in goat plasma (25). We did not detect it in goats with the methods used in the present study.

Palmitic and oleic acids have the lowest variability in percent composition throughout exercise and recovery (Table 2). Therefore, changes in their concentrations are the most representative of changes in total plasma NEFA, and they should be the preferred labeled fatty acids for flux measurements in dogs and goats. Also, both of these acids are relatively abundant, making the measurement of their specific radioactivity or enrichment easier than with other much less concentrated NEFA. Palmitic and oleic acids have been used extensively in NEFA kinetics studies in humans (12,13,27), and the present study suggests that they are also suitable for such studies in other mammalian species, even during exercise.

ACKNOWLEDGMENTS

This work was supported by an NSERC grant (Canada) to J.-M.W. (OGP0105639) and an NSF grant to C.R.T. (DCB8918371). G. McC. was the recipient of a fellowship from the French Ministry of External Affairs (CIES).

REFERENCES

1. Newsholme, E.A. (1988) in *Principles of Exercise Biochemistry* (Poortmans, J.R., ed.) pp. 40–77, Karger, Basel.
2. Hochachka, P.W., and Somero, G.N. (1984) *Biochemical Adaptation,* pp. 85–144, Princeton University Press, Princeton.
3. Roberts, T.J., Weber, J.-M., and Taylor, C.R. (1990) *The Physiologist 33,* A110.
4. McClelland, G., Zwingelstein, G., Taylor, C.R., and Weber, J.-M. (1994) *Am. J. Physiol. 266,* R1280–R1286.
5. Weber, J.-M., Roberts, T.J., and Taylor, C.R. (1993) *Am. J. Physiol. 264,* R797–R803.
6. Wolfe, R.R., Klein, S., Carraro, F., and Weber, J.-M. (1990) *Am. J. Physiol. 258,* E382–E389.
7. Nakamura, H., Faludi, G., and Spitzer, J.J. (1967) *Diabetes 16,* 175–180.
8. Taylor, C.R., Karas, R.H., Weibel, E.R., and Hoppeler, H. (1987) *Resp. Physiol. 69,* 1–27.
9. Dziuk, H.E. (1984) in *Dukes' Physiology of Domestic Animals* (Swenson, M.J., ed.) pp. 320–339, Cornell University Press, Ithaca.
10. Argenzio, R.A. (1984) in *Dukes' Physiology of Domestic Animals* (Swenson, M.J., ed.) pp. 301–310, Cornell University Press, Ithaca.
11. Jéquier, E. (1992) in *Energy Metabolism: Tissue Determinants and Cellular Corollaries* (Kinney, J.M., and Tucker, H.N., eds.) pp. 123–139, Raven Press, New York.
12. Spitzer, J.J. (1975) *Fed. Proc.* 34, 2242–2245.
13. Hagenfeldt, L. (1975) *Fed. Proc. 34,* 2236–2240.
14. Fredrickson, D.S., and Gordon, R.S. (1958) *J. Clin. Invest. 37,* 1507–1515.
15. Paul, P., and Issekutz, J.B. (1967) *J. Appl. Physiol. 22,* 615–622.

16. Fedak, M.A., Rome, L., and Seeherman, H.J. (1981) *J. Appl. Physiol. 51,* 772–776.
17. Bahr, R., Hostmark, A.T., Newsholme, E.A., Gronnerod, O., and Sejersted, O.M. (1991) *Acta Physiol. Scand. 143,* 105–115.
18. Tserng, K.-Y., Kliegman, R.M., Miettinen, E.-L., and Kalham, S.C. (1981) *J. Lipid Res. 22,* 852–858.
19. Zar, J.H. (1984) *Biostatistical Analysis,* 2nd edn., p. 32, Prentice-Hall, Englewood Cliffs.
20. Havel, R.J., Carlson, L.A., Ekeland, L.G., and Holmgren, A. (1964) *J. Appl. Physiol. 19,* 613–618.
21. Hagenfeldt, L., Wahren, J., Pernow, B., and Ekestrom, S. (1972) *J. Clin. Invest. 51,* 3061–3071.
22. Carlson, M.G., Snead, W.L., Hill, J.O., Nurjhan, N., and Campbell, P.J. (1991) *Am. J. Physiol. 261,* E815–E820.
23. Lin, K.-C., Cross, H.R., and Smith, S.B. (1992) *Lipids 27,* 111–116.
24. Payne, E., and Masters, C.J. (1971) *Int. J. Biochem. 2,* 623–643.
25. Leat, W.M.F., and Baker, J. (1970) *Comp. Biochem. Physiol. 36,* 153–161.
26. Goodwin, T.W., and Mercer, E.I. (1983) *Introduction to Plant Biochemistry,* 2nd edn., p. 275, Pergamon Press Ltd., New York.
27. Wolfe, R.R. (1984) *Tracers in Metabolic Research. Radioisotope and Stable Isotope/Mass Spectrometry Methods,* p. 287, Alan R. Liss, New York.

[Received December 9, 1993, and in revised form December 16, 1994; Revision accepted December 28, 1994]

Effect of Reproductive States on Lipid Mobilization and Linoleic Acid Metabolism in Mammary Glands

Gautam K. Bandyopadhyay, Le-Young Lee, Raphael C. Guzman and Satyabrata Nandi*

Cancer Research Laboratory, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

ABSTRACT: Effects of pregnancy and lactation on lipid metabolism in mouse mammary fat pads and nonmammary adipose tissues have been studied. In order to address the question whether the influence of hormonal milieu on lipid metabolism in mammary epithelial cells during pregnancy and lactation is the same as in fat cells, we have studied the mobilization of lipids and metabolism of fatty acids in the intact mammary glands, parenchyma-free mammary fat pads and in the perimetrial fat tissues of virgin, pregnant and lactating mice. Compared to parenchyma-free mammary fat pads, the perimetrial adipose tissues accumulated 5-fold higher levels of triglycerides during pregnancy. Mammary fat cells maintained overall lipid levels during pregnancy and lactation (16–20 μg/fat pad). In contrast, lactation depleted total lipid stores from 108 ± 5 to 24 ± 4.5 μg/fat pad in perimetrial fat pads. Results of comparative analysis of fatty acid composition of mammary fat pads, with and without epithelial tissue, from virgin and lactating mice showed stimulation of 18:2ω6 metabolism leading to 130% increase in the ratio 20:4ω6 to 18:2ω6 in the epithelial compartment. Pregnancy and lactation resulted in the elevation of 20:4ω6 levels probably due to a 4-fold increase in Δ5 desaturase activity and a decrease in oxidative degradation of 18:2ω6. These results suggest that, unlike other adipose tissues, the metabolic pathways in mammary fat cells are not dedicated to sequestration and accumulation of dietary lipids during pregnancy. Lactation favors mammary epithelial cell-stimulated production of precursors of eicosanoids which are known to have agonist-like effect on mammary epithelial cells.

Lipids 30, 155–162 (1995).

The production of milk by the mammary glands is controlled by multiple hormones (1,2). These lactogenic hormones not only regulate the expression of mammary-specific products, but also prepare other organs and tissues to support milk production (3). In response to the preparations for lactation, mammary tissues undergo metabolic changes, many of which are reciprocal to the corresponding changes in the adipose tissues.

During lactation, the uptake of circulating lipids and carbohydrates and the synthesis of lipids in mammary tissue are increased, whereas the converse is true for nonmammary adipose tissue. During pregnancy and before parturition, adipose tissue accumulates more lipids than mammary glands; however, as lactation ensues, the metabolic pattern is switched (3–9). It has been suggested (see Ref. 3 for a review) that the modulation of insulin sensitivity of tissues by progesterone and prolactin plays a role in this regulation.

The metabolic interaction between mammary and adipose tissues may help in supplying essential fatty acids (EFA) and other nutrients from the depot fat to the mammary glands. Dietary EFA can be received from the circulation, but these fatty acids are turned over and metabolized. By comparison, adipose tissues can store EFA (10–12), and, therefore, these fatty acids will be available even when the dietary supply is reduced. It has been shown previously that dietary EFA deficiency affects the maintenance and induction of prolactin receptors in liver (13) and arrests mammary development in young mice (14). Dietary linoleate, but not saturated fats, can accelerate the growth and branching of mammary ducts in young mice (15). Such influences of EFA on mammary gland development are partly dependent on age and maturation of the ovary (16).

What is not clear from most of these studies is the role of mammary fatty tissue of the gland, which surrounds the epithelial tissue but is separated from it by the basement membrane. Previous studies have demonstrated that mammary epithelial tissue can develop and function normally when transplanted to other white adipose tissue (17–19). Results from two laboratories have also suggested that local stromal environment has the capacity to influence mammary epithelial response to hormones and that estrogen stimulated mammary epithelial growth may be mediated through its actions on surrounding adipocytes (20–22). While the fat cells in a gland can influence the functions of epithelial cells, the presence of epithelial cells in a fat pad can also modify the response of fat cells to some agonists. Thus, in pregnant swine, response to norepinephrine leading to fatty acid release was found to be higher in mammary fat pads than that in peritoneal fat pads (23).

In this context, our interests were to study (i) the paracrine role of lipids in the interactions between stromal adipocytes and epithelial cells of mammary glands, and (ii) the fate of

*To whom correspondence should be addressed at Cancer Research Laboratory, 491 Life Sciences Addition, University of California, Berkeley, CA 94720-2751.

Abbreviations: AFP, abdominal fat pad; CMFP, cleared mammary fat pad; DG, diglycerides; DMEM, Dulbecco's modified Eagle's medium: EFA, essential fatty acids; HPLC, high-performance liquid chromatography; IMFP, intact mammary fat pad; LPL, lipoprotein lipase; PL, phospholipids; TG, triglycerides; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

linoleic acid, which is stored in the fat cells and can be transported into the epithelial cells, in response to hormonal changes during pregnancy and lactation. Earlier studies from our and other laboratories have demonstrated that EFA, such as 18:2ω6 and 20:4ω6, can directly influence the epidermal growth factor and hormonal effects on mammary epithelial proliferation and differentiation (24–27). In the present study we have used mammary fat pads free of epithelial tissue as representative of mammary fat cells in mice. By comparing the lipid composition of cleared fat pads and intact mammary glands under different reproductive states, we report here that, unlike other adipose tissues, mammary fat cells do not accumulate lipids during pregnancy. Lactation-induced biochemical changes in the mammary glands allow mammary tissues to maintain higher levels of 18:2ω6 and 20:4ω6 compared to mammary tissues in virgin mice.

MATERIALS AND METHODS

Materials. Reference fatty acids were purchased from Biomol (Plymouth Meeting, PA) and Ocean Organics Inc. (Peace Dale, RI). [1-^{14}C]18:2ω6 and [1-^{14}C]20:3ω6 were purchased from New England Nuclear (Boston, MA). High-performance liquid chromatography (HPLC)-grade solvents and other chemical reagents were bought from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO), respectively. Culture media were obtained from GIBCO-BRL (Gaithersburg, MD). Ovine prolactin (NIAMDD o-Prl) was a gift from the National Institutes of Health (Bethesda, MD).

Animal procedures. Female Balb/C mice were obtained from the animal colony maintained by the Cancer Research Laboratory, University of California (Berkeley, CA). One group of 6-week-old and another group of 8-week-old virgin female mice, 12 mice in each group, were allowed to mate with males, and the formation of vaginal plugs was examined each morning to determine pregnancy. The older pregnant mice were sacrificed by cervical dislocation on day 11 of pregnancy (midpregnant). The younger group of pregnant mice were allowed to give birth to pups (after 18–19 d of pregnancy) and lactate. At day 10 of lactation, pups were removed and mothers were sacrificed. Virgin mice of same age served as control. At the time of sacrifice, virgin, pregnant and lactating mice were 70–72 days old. For collection of number 4 inguinal mammary fat pads, the mice were killed by cervical dislocation, the lymph nodes were removed and tissues were collected in a tube containing medium 199 (GIBCO-BRL). Mammary tissues were sliced with a scalpel to 1–2 mm thickness and washed free of fluids (e.g., milk from the lactating tissue) by incubation (each time for 10 min) and rinsing five times with medium 199. Abdominal fat pads were obtained from the fatty tissues surrounding the uterus (perimetrial fat pad) and were similarly sliced and washed. The perimetrial adipose tissue was used as a representative of abdominal fat pads.

All mice were fed *ad libitum* with food pellets (Wayne rodent blox 8656) which contained 5% (w/w) fat contributed by dry yeast, soybean oil, animal liver and fish meal. The fatty acid composition of this diet is given in Table 1.

Mammary parenchyma from one of the two number 4 inguinal fat pads was removed from eighteen 3-week-old mice following a procedure described in detail by DeOme *et al.* (28). Mice were anesthetized by injecting (10 μL/g body weight) a 10% (vol/vol) Nembutal solution (Abbott Laboratories, Chicago, IL) prepared in a solvent mixture composed of normal saline/propylene glycol/ethanol (6:2:1, vol/vol). An inverted Y-shaped skin incision (1 inch long) was made along the midline of the abdomen and between the inguinal and perianal nipples along the hind legs (½ inch long). The exposed inguinal nipple of one number 4 mammary fat pad and the large blood vessel running from the lymph node were cauterized. The areas of the number 4 fat pad containing growing ductal elements are dissected free of skin and discarded. The

TABLE 1
Fatty Acid Composition of Mammary and Adipose Tissues of Virgin, Pregnant and Lactating Mice[a]

	10:0	12:0	14:0	16:0	18:0	16:1ω7	18:1ω9	18:2ω6	18:3ω6	20:3ω6	20:4ω6
						(mol%)					
Diet[b]	2.0 ± 0.3	2.1 ± 0.4	3.5 ± 0.6	17.7 ± 2.1	3.7 ± 0.5	3.3 ± 0.4	19.2 ± 1.9	43.4 ± 3.8	0.8 ± 0.2		
Virgin											
IMFP	1.2 ± 0.4	0.4 ± 0.5	3.4 ± 0.8	25.4 ± 2.0	5.1 ± 0.8	6.0 ± 1.2	28.4 ± 3.1	28.1 ± 3.0	1.3 ± 0.3	0.5 ± 0.1	5.8 ± 0.5
CMFP	0.7 ± 0.1	0.5 ± 0.1	4.7 ± 0.4	25.2 ± 0.7	3.7 ± 0.6	5.6 ± 0.7	32.1 ± 4.0	26.3 ± 1.6	1.3 ± 0.2		
AFP	1.4 ± 0.7	0.4 ± 0.1	2.5 ± 0.3	25.1 ± 3.1	2.8 ± 1.0	4.2 ± 0.2	33.6 ± 1.9	29.0 ± 6.0	1.2 ± 0.2		
Midpregnant											
IMFP	1.8 ± 0.7	0.8 ± 0.2	3.0 ± 0.7	24.2 ± 3.0	3.9 ± 0.5	6.3 ± 1.3	30.0 ± 5.0	19.8 ± 1.3[c]	0.9 ± 0.3	0.54 ± 0.1	8.2 ± 1.3[c]
CMFP	1.3 ± 0.5	1.4 ± 0.5	2.8 ± 0.3	25.8 ± 3.4	5.8 ± 1.4	4.8 ± 0.2	30.7 ± 4.4	26.0 ± 1.9	1.3 ± 0.1		
AFP	0.7 ± 0.1	0.5 ± 0.1	2.6 ± 0.6	5.7 ± 0.6	2.2 ± 0.5	6.5 ± 0.6	39.5 ± 3.3	40.2 ± 1.2	2.1 ± 0.3		
Lactating											
IMFP	10.0 ± 1.8	13.1 ± 1.3	9.0 ± 1.4	15.4 ± 3.1	3.1 ± 1.1	3.1 ± 0.4	17.5 ± 0.5	16.6 ± 1.5[c]	2.0 ± 0.4	1.4 ± 0.15	8.8 ± 0.9[c]
CMFP	1.3 ± 0.1	1.05 ± 0.2	3.0 ± 0.3	24.2 ± 2.3	7.4 ± 1.0	5.0 ± 0.6	37.3 ± 4.0	19.5 ± 1.5[c]	0.5 ± 0.1		
AFP	2.3 ± 1.0	0.7 ± 0.1	2.8 ± 0.6	24.4 ± 2.4	4.4 ± 0.8	6.9 ± 0.6	37.0 ± 4.5	17.8 ± 3.0[d]	3.6 ± 0.1		

[a]Results are presented as the mean SE for five replicate samples. Abbreviations: AFP, abdominal fat pad; CMEP, cleared mammary fat pad; IMFP, intact mammary fat pad. [b]The other fatty acid components of the diet are 18:3ω3 (2.5 ± 0.3) and 20-5ω3 (1.1 ± 0.2). [c]Significantly different ($P < 0.05$) from the corresponding tissues in virgin mice. [d]Difference from virgin mice was not significant.

remaining gland-free mammary fat pad, called cleared mammary fat pad, is filled with fat cells (along with other stromal components) but is devoid of mammary epithelial tissue. The two types of contralateral number 4 fat pads from the same animal, one with and the other without the epithelial tissue, and exposed to the same hormonal milieu, offer a good system for comparison of the effects of hormones on epithelial vs. fatty tissues of the mammary glands. Twelve mice with cleared mammary fat pads were mated to produce pregnant and lactating mice. In all experiments, five mice were used for each experimental group representing a physiological state. Tissues from each mouse were analyzed separately.

Explant culture and incorporation of labeled fatty acids. Fragments of number 4 inguinal mammary fat pads (*ca.* 1.0 cubic mm in size and *ca.* 1.0 mg in weight) were prepared by following the procedure described by Topper *et al.* (29). Mammary explants were transferred onto triangular-shaped steel-wired grids (15 explants per grid) and placed inside 6-well culture plates covered with medium (3 mL). Corners of the grids were bent so that the grids could stand as raised platforms, and explants on them could remain close to the air/liquid interface. Explants were cultured for 2 d in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, vol/vol; GIBCO-BRL) containing delipidated bovine serum albumin (1 mg/mL), soybean trypsin inhibitor (5 μg/mL), transferrin (5 μg/mL), Vitamin E (1 μg/mL), amphotericin B (2 μg/mL), penicillin (30 μg/mL), streptomycin sulfate (50 μg/mL) and a trace element cocktail composed of (final concentration) 1 nM $MnCl_2$, 10 nM H_2SeO_3, 50 μM Na_2SiO_3, 1 nM $(NH_4)_6Mo_7O_{24}$, 5 nM NH_4VO_3, 0.5 nM $NiCl_2$, and 0.5 nM $SnCl_2$. During the first 24 h in culture, the medium was supplemented with insulin (100 ng/mL). For the next 24 h, explants were cultured in the presence of radiolabeled fatty acids (as bovine serum albumin conjugates, 2.0 μg Ci/mL) in medium supplemented with insulin (100 ng/mL), prolactin (1 μg/mL) and cortisol (100 nM). After 24 h of incubation, the labeled media were removed, and the explants were washed with chilled unlabeled medium and then frozen.

Lipid extraction, separation and analysis. The detailed procedures for lipid extraction and separation by thin-layer chromatography (TLC) have been described previously (25). Tissues were first homogenized by Polytron (Brinkmann Instruments, Switzerland) in 0.8 ml ± HCl (0.1 N) and then blended with 3 mL of a mixture of chloroform/methanol (1:2, vol/vol). The monophasic mixture was centrifuged, and the clear supernatant was mixed with an equal volume of 1% NaCl solution and chloroform. The mixture was vortexed and centrifuged to separate two solvent layers. The lower organic layer containing tissue lipids was collected, and the extraction was repeated with the top aqueous layer. The organic phase was dehydrated by anhydrous sodium sulfate and concentrated by blowing nitrogen gas. An aliquot was subjected to TLC analysis for neutral lipids (developed inside a fume hood using a solvent mixture: hexane/diethyl ether/acetic acid, 70:30:1, by vol) and phospholipids (developing solvent: chloroform/methanol/acetone/acetic acid/water, 6:2:8:2:1, by vol). Another aliquot of the lipid extract was digested in 5% methanolic KOH solution and then acidified to generate free fatty acids. Free fatty acids were converted to bromophenacyl derivatives and analyzed by HPLC as described previously (30). Quantitations of total triglycerides, diglycerides and phospholipids were done by assaying total glycerol (after saponification), acyl esters (31,32) and organic phosphates (33) in the extracts of TLC separated lipids. Glycerol was assayed by using a kit from Boehringer Mannheim (Indianapolis, IN).

Determination of Δ5 desaturase activity. Microsomes were prepared and conversion of [1-^{14}C]20:3ω6 to [1-^{14}C]-20:4ω6 by microsomal Δ5 desaturase activity was determined by following the procedure described by Mandon *et al.* (34). Labeled fatty acids were analyzed by HPLC and monitored by Flow-One Beta radiodetector (Radiomatic Instruments, Tampa, FL).

Presentation of results. To compare the effect of reproductive states on lipid metabolism in three different tissues, groups of virgin, pregnant and lactating mice (five mice per group maintained on the same diet) were sacrificed when they were 70–72 days old. The group of virgin mice served as control. Data are presented as means ± SE for five replicate samples, each obtained from one mouse. Two such experiments were carried out, and the results from one experiment are presented. The significance of the differences in the data was tested by Wilcoxon's rank sum test at the 5% level. In some experiments, duplicate samples were analyzed and mean of the duplicates was presented. In these cases, data from two separate experiments were pooled for analysis of significance of differences in the data.The data obtained from the glands under one physiological state have been compared with the glands from another physiological state on the basis of differences observed in the total levels during the change of physiological states. This comparison reflects the total amount of change that occurred per gland when virgin mice went through pregnancy and lactation. Since the wet weight, DNA and protein contents of mammary glands and adipose tissues did not remain constant during these changes in physiological states, the normalization of data with respect to these parameters did not represent the true comparison of the metabolic alterations.

RESULTS

Pregnancy and lactation in mice caused an increase in the amount of total DNA and protein in intact mammary fat pads (IMFP). Compared to nonpregnant tissue, there was a 2-fold increase in DNA and protein content at midpregnancy, and a 6- to 7-fold increase in DNA and an 8-fold increase in protein by the tenth day of lactation (Table 2). The DNA content in the epithelial tissue alone was estimated by subtracting the total amount of DNA in cleared mammary fat pads (CMFP) from that of IMFP. The increase in total DNA content in the epithelial tissue of IMFP of lactating mice was 10-fold higher than IMFP of virgin mice. This value was lower than the previously reported estimation where the increase was 20- to 25-

TABLE 2
Total Protein and DNA Levels of Mammary and Adipose Tissues of Mice Under Different Reproductive Conditions[a]

Physiological condition	Tissue	DNA (μg/fat pad)	Protein (mg/fat pad)
Virgin	IMFP	60 ± 7	2.34 ± 0.5
	CMFP	23 ± 3	1.6 ± 0.15
	AFP	250 ± 33	4.8 ± 0.31
Midpregnant	IMFP	130 ± 21	4.1 ± 0.21
	CMFP	430 ± 10	1.74 ± 0.11
	AFP	300 ± 36	5.3 ± 0.33
Lactating	IMFP	400 ± 80	17.3 ± 3.5
	CMFP	14 ± 1	1.10 ± 0.05
	AFP	53 ± 14	4.3 ± 0.47

[a]Results are presented as the mean ± SE per fat pad to indicate the extent of changes that occurred in the whole tissue. Five replicate samples per group were analyzed. Five mice, each providing one sample fat pad for analysis, were used per group. For other details, see the legend of Figure 1 and the Materials and Methods section. For abbreviations, see Table 1.

fold (35). CMFP and abdominal fat pads (AFP) under similar conditions did not show these changes. Rather, a significant decrease in DNA content was observed which cannot be explained at this stage. However, lipid accumulation was different in CMFP and AFP. While lipid levels in AFP increased dramatically during pregnancy, CMFP and IMFP did not show such trends (Fig. 1A). In lactating mice, the lipid content of IMFP was 2- to 3-fold higher than CMFP (Fig. 1A), suggesting that lipogenesis was taking place in the epithelial compartment of IMFP. These increases in lipid content were contributed by triglycerides (TG) in AFP (Fig. 1B) and by triglycerides and phospholipids (PL) in IMFP (Fig. 1B and D). The higher diglyceride (DG) levels in AFP (Fig. 1C) may have contributed to increased accumulation of TG. Lactation caused depletion of TG store in AFP (Fig. 1B), but TG levels in the lactating IMFP were similar to AFP. Probably, lactating IMFP channeled most of the TG into secreted milk and could not store more TG than other tissues. The rise in PL in the lactating IMFP (Fig. 1D) corresponded to the increased number of epithelial cells containing higher levels of endoplasmic reticulum and secretory vesicles.

The results of the analyses of fatty acid composition of lipids from mammary and adipose tissues are shown in Tables 1 and 3. The fatty acid profile of lactating IMFP, which actually represents epithelial tissue plus residual milk lipids, showed an expected rise in the level of medium-chain fatty acids (10:0,12:0,14:0) probably due to the induction of medium-chain acyl thioesterase activity (5,6). A comparison of the levels and the composition of fatty acids in the tissues showed that, compared to virgin mice, there was a 50% and a 130% rise in the ratio 20:4ω6 to 18:2ω6 in pregnant and lactating mice, respectively (Table 3). The rise in 20:4ω6 level was not observed in CMFP and AFP (Tables 1 and 3) under similar hormonal and physiological conditions.

Pregnancy increased the total lipid content in AFP and led to the building up of a large store of 16:1ω7, 18:1ω9 and

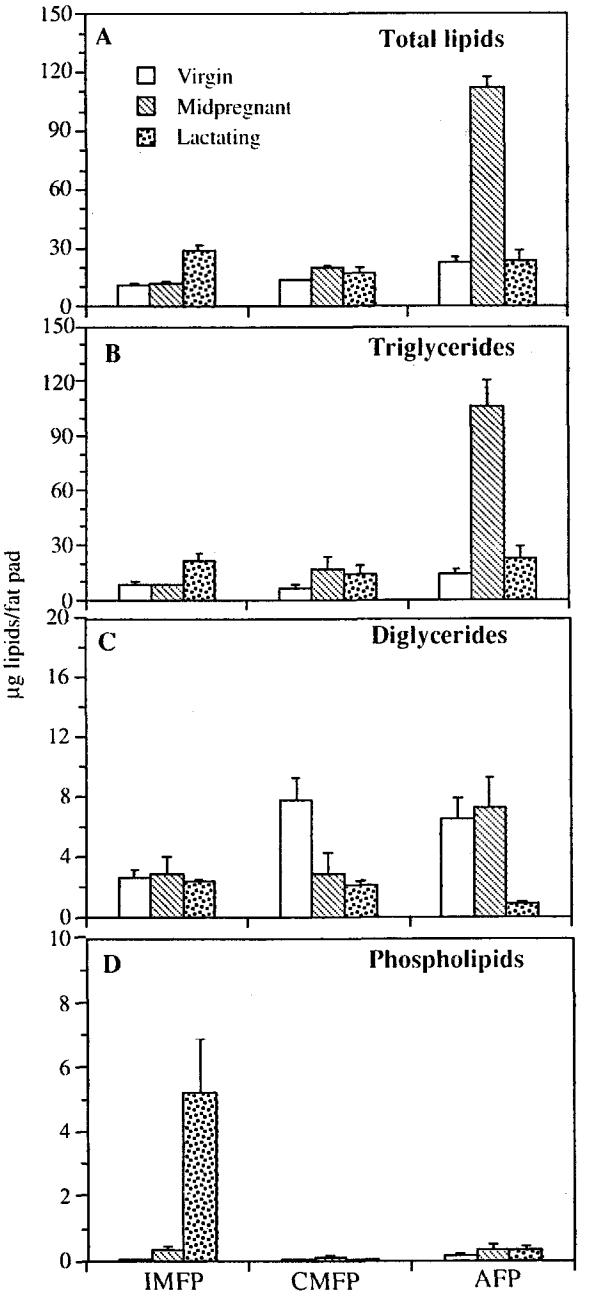


FIG. 1. Changes in total lipid, triglyceride, diglyceride and phospholipid levels in intact mammary fat pads (IMFP), cleared mammary fat pads (CMFP) and abdominal fat pads (AFP) from virgin, pregnant and lactating mice. Results are presented as mean ± SE per fat pad to indicate the extent of changes that occurred in the whole tissue. The entire number 4 inguinal mammary fat pads and perimetrial fat pads (representing abdominal fat pads) were collected for analysis. Five replicate samples per group were analyzed. Five mice, each providing one sample fat pad for analysis, were used per group. For analytical details, see the Materials and Methods section.

18:2ω6. This supply was depleted during lactation, bringing the level down to that similar to virgin mice (Table 3). In this respect, the metabolic response of CMFP was different from that of AFP. There was neither a decrease in the saturated fatty acid composition nor an increase in the accumulation of

TABLE 3
Fatty Acid Composition of the Total Lipids from Mammary Fat Pads, with and without Epithelial Tissue, and Adipose Tissues of Virgin, Pregnant and Lactating Mice[a]

Fatty acids	Virgin mice			Midpregnant mice			Lactating mice		
	IMFP	CMFP	AFP	IMFP	CMFP	AFP	IMFP	CMFP	AFP
	(nmol fatty acid/fat pad)								
10:0	0.47 ± 0.2	0.31 ± 0.1	1.05 ± 0.5	0.84 ± 0.3	0.85 ± 0.3	3.0 ± 0.2	9.7 ± 1.8	0.8 ± 0.1	1.9 ± 0.8
12:0	0.14 ± 0.1	0.23 ± 0.1	0.3 ± 0.1	0.37 ± 0.1	0.87 ± 0.3	1.85 ± 0.4	12.7 ± 1.3	0.5 ± 0.2	0.6 ± 0.1
14:0	1.3 ± 0.3	2.2 ± 0.2	1.8 ± 0.2	1.4 ± 0.34	1.8 ± 0.17	10.2 ± 2.5	8.6 ± 1.4	1.8 ± 0.2	2.3 ± 0.5
16:0	9.8 ± 0.8	11.8 ± 0.3	18.6 ± 2.3	11.2 ± 1.4	16.5 ± 2.2	22.7 ± 2.1	15.0 ± 3.0	14.0 ± 1.4	20.0 ± 2.0
18:0	2.0 ± 0.3	2.6 ± 0.3	2.1 ± 0.8	1.8 ± 0.23	3.7 ± 0.9	8.85 ± 2.0	3.1 ± 1.1	4.0 ± 0.6	3.6 ± 0.7
16:1ω7	2.3 ± 0.4	1.7 ± 0.3	3.1 ± 0.1	2.9 ± 0.6	3.1 ± 0.16	25.7 ± 2.3	3.0 ± 0.4	3.2 ± 0.4	5.7 ± 0.5
18:1ω9	11.1 ± 1.2	15.0 ± 1.8	25.0 ± 1.5	13.8 ± 2.3	19.6 ± 2.8	157.2 ± 13.0	17.0 ± 0.5	21.0 ± 2.4	30.3 ± 3.7
18:2ω6	10.9 ± 1.2[b]	12.6 ± 0.8	21.5 ± 4.5	9.1 ± 0.6	16.7 ± 1.2	160.0 ± 5.0	16.1 ± 1.5[b]	11.0 ± 0.9	14.6 ± 2.5
18:3ω6	0.52 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.4 ± 0.15	0.83 ± .1	8.4 ± 1.0	1.9 ± 0.4	0.3 ± 0.3	3.0 ± 0.1
20:3ω6	0.20 ± 0.1			0.25 ± 0.1			1.4 ± 0.1		
20:4ω6	2.5 ± 0.2[b]			3.2 ± 0.6			8.5 ± 0.9[b]		
20:4ω6/18:2ω6	0.23			0.35			0.53		

[a]Results from one of the two experiments are presented as the mean ± SE for five replicate samples, each obtained from one mouse. The two experiments showed similar trends. For abbreviations, see Table 1.
[b]The amounts of 18:2ω6 and 20:4ω6 in lactating mice were significantly ($P < 0.05$, Wilcoxon test) higher than in virgin or pregnant mice.

unsaturated fatty acids in CMFP of pregnant mice or the mobilization of unsaturated fatty acids in CMFP of lactating mice (Tables 1 and 3).

It should be noted that the results in Table 3 are presented as nmol per fat pad to indicate the level of fatty acids, and those in Table 1 are presented as mol% composition. A comparison of the composition of dietary and tissue fatty acids demonstrated that the proportion of monounsaturated fatty acids in the mammary and adipose tissues was higher than that of the diet, whereas the opposite was true for 18:2ω6 except in AFP of pregnant mice where the percentage of 18:2ω6 was comparable to that in the diet. The proportions of saturated fatty acids in AFP of pregnant mice were considerably lower than those in other tissues (Table 1) mainly due to a decrease in the percentage of 16:0. In pregnant mice, the mobilization of 18:2ω6 in IMFP was similar to that of lactating mice, but CMFP maintained a similar proportion of 18:2ω6 as in virgin mice. The percentage of 18:2ω6 in the lactating mice was significantly ($P < 0.05$) lower than that of virgin mice. IMFP and CMFP of lactating mice showed a similar pattern and differed from AFP where the proportion of 18:2ω6 was not significantly different from that of virgin mice. Although the percent composition of 16:0 in AFP of pregnant mice and 18:2ω6 in IMFP and CMFP of lactating mice were lower than that of virgin mice (Table 1), the total level of these fatty acids in those tissues did not show any decrease (Table 3).

The metabolism of 18:2ω6 in the IMFP explants *in vitro* demonstrated that, compared to the explants from virgin mice, there were 100 and 300% increases in the production of 20:3ω6 and 20:4ω6, respectively, from labeled 18:2ω6 by the mammary explants of pregnant and lactating mice (Table 4). Parallel to these changes in linoleate metabolism, microsomal Δ5 desaturase activity was also found to be 4-fold higher in the tissues from pregnant and lactating mice compared to virgin mice (Table 4). In mouse liver lipids, the ratio of 20:4ω6 to 18:2ω6 in the pregnant tissues (0.75 ± 0.2) was not significantly different from virgin tissue (0.6 ± 0.1), suggesting that pregnancy did not affect linoleate metabolism in liver dramatically (results not shown).

In contrast to their lower capacities for desaturation, the IMFP of virgin mice had a much higher capacity for oxidative degradation of incorporated [1-^{14}C]18:2ω6 than the IMFP of lactating mice (Table 4). While the IMFP of lactating mice actively desaturated and chain-elongated 18:2ω6 to produce 18:3ω6, 20:3ω6 and 20:4ω6, there was little radioactivity in the saturated and monounsaturated fatty acids. On the other hand, when virgin IMFP explants were incubated under similar conditions, labeling of saturated fatty acids occurred but remained restricted to 10:0 and 12:0 fatty acids (Table 4). In addition, 18:3ω6, 20:3ω6 and some 20:4ω6 were also produced.

DISCUSSION

Pregnancy and lactation change the pattern of lipid metabolism as a whole in adipose tissue and mammary gland. During pregnancy, adipose tissue has the highest accumulation of TG, but the TG store is depleted during lactation, as we have observed in AFP. Our results demonstrated that fat cells from AFP and CMFP behaved differently during pregnancy. In contrast to AFP, pregnancy and lactation did not cause dramatic changes in the lipid levels in CMFP. Since changes in the concentration of DG and TG in CMFP were moderate and reciprocal in nature, overall levels of esterified lipids were maintained during pregnancy and lactation.

A number of factors, including the elevated levels of circulating progesterone (36–38) and insulin (39,40) and higher

TABLE 4
Incorporation and Metabolism of [1-^{14}C]18:2ω6 in the Explant Cultures of Mammary Glands Obtained from Virgin, Pregnant and Lactating Mice[a]

Reproductive states	Incorporation into cellular lipids (% of labeled fatty acid added)[a]	Major labeled fatty acids					Microsomal Δ5 desaturase activities (units)[c]
		Saturated[b]	18:2ω6	18:3ω6	20:3ω6	20:4ω6[e]	
		(% of radioactivity incorporated)					
Virgin	8.0, 6.4	505, 575[d]	30.0, 25.5[d]	3.4, 2.1	8.6, 10.3	1.2, 1.0[d]	2.1 ± 1.0[d]
Midpregnant	8.2, 8.6	24.0, 12.5	43.1, 53.2	0.5, 0.1	17.9, 18.1	4.8, 4.5	8.7 ± 0.5
Lactating	8.8, 9.6	2.0, 1.5	57.7, 57.2[e]	2.0, 1.2	18.0, 21.3	6.0, 4.6[e]	8.4 ± 0.6[e]

[a]The results from the two incorporation experiments, each carried out in duplicate, have been presented as a mean of the duplicate assays. Both mean values from two experiments are shown. The four datum points (from two duplicate assays) in one category were compared with the others, and the significance of differences was tested. Incorporations were normalized against 15 mg wet weight for each tissue (total tissue weight per well).
[b]Saturated fatty acids represent $C_{10.0}$–$C_{12.0}$ region of the HPLC chromatogram.
[c]1 unit = p mols of [1-^{14}C]20:3ω6 converted to [1-^{14}C]20 4ω6/mg microsomal protein/15 min at 30° ± SE (three experiments).
[d]Significantly different ($P < 0.05$) from pregnant and lactating mice.
[e]Differences from midpregnant mice are not significant.

lipoprotein lipase (LPL) activity (41,42) in the tissue, have been found to contribute to the accumulation of TG in adipose tissue. During lactation, lipogenesis in the mammary gland is stimulated with a corresponding decline in nonmammary adipose tissue (3,6,43,44). It appears that the effect of progesterone on CMFP is different from that on AFP and the lack of LPL activity in the fat cells of mammary tissues, as shown by Camp *et al.* (45), may be responsible for a lower level of TG accumulation in CMFP during pregnancy. Our data suggest that mammary fat cells may differ from nonmammary fat cells (or other adipose tissues) in sequestering dietary lipids and delivering them to lactating mammary epithelial tissue.

The fatty acid composition revealed that pregnancy caused a large accumulation of monounsaturated fatty acids and 18:2ω6 in the lipid stores of AFP which were mobilized during lactation. Unlike AFP, the level of 18:2ω6 in CMFP of pregnant mice was same as in virgin mice. But the fat cells of mammary tissue can transport free fatty acids (produced by lipolysis of TG by hormone sensitive lipase and released extracellularly due to lack of reesterification) directly across the basement membrane into the epithelial tissue without going into circulation. Therefore, stored linoleate of mammary fat cells, even though the store is not as large as in AFP, can be an important source of EFA for mammary epithelial cell functions.

The percentage of 18:2ω6 in the IMFP of lactating mice was low compared to virgin mice due to higher desaturation and chain elongation of 18:2ω6 and accumulation of medium chain fatty acids in the lactating mice. However, as the level of total lipids in the IMFP of lactating mice was much higher than that of virgin mice, the total amount of 18:2ω6 was also maintained at a higher level in the lactating mice. It appears that the decrease in percent composition of fatty acids was compensated for by an increase in accumulation of total lipids (therefore, higher total fatty acid levels) in respective tissues.

The contribution of 18:2ω6 in the development, proliferation and differentiation of mammary epithelial cells has been documented (24–27). Dietary 18:2ω6, stored mostly as TG in the fat cells, can be released into circulation under the influence of hormones and utilized by mammary epithelial cells. The metabolism of 18:2ω6 by the mammary cells is influenced by physiological conditions. Proliferating epithelial tissue in the pregnant and lactating mice has elevated levels of Δ5 desaturase activity, and, as a result, the IMFP of lactating mice are able to maintain a higher level of 20:4ω6. Cleared mammary fat pads in the lactating mice (contralateral to IMFP) did not show higher 20:4ω6 levels. Therefore, it can be inferred that increased metabolism of linoleate through Δ5 desaturation occurred in the epithelium compartment. Work by others has demonstrated that fat-free diets cause decreased Δ5 desaturase activity in liver microsomes and that supplementation of ω6-unsaturated fatty acids in the diet restored the activity level (46,47). Our results emphasize that in the mammary glands under similar dietary regimen, differentiation of epithelial tissue (as in pregnancy and lactation) raises Δ5 desaturase activity in the epithelial compartment of IMFP.

Although the higher desaturase activity may be partly accounted for by higher epithelial cellularity in the mammary tissues of pregnant and lactating mice (thus contributing higher amounts of epithelial proteins) compared to virgin mice, calculations based on protein content show that while the amount of protein in the IMFP of pregnant mice was two times higher than that of virgin mice, there was a 4-fold increase in Δ5 desaturase activity in the tissues of pregnant mice over virgin mice. This suggests an induction or activation of desaturase activity during pregnancy. Similar consideration for accounting desaturase activity in the IMFP of lactating mice cannot be made because IMFP of lactating mice contains a large amount of casein vesicles. This contributes to IMFP of lactating mice having seven times more protein than that of virgin mice, whereas Δ5 desaturase activity was 4-fold higher. Because microsomal preparations from IMFP of lactating mice are likely to contain large proportions of secretory casein resides, true comparisons of specific activities

of Δ5 desaturase between IMFP of virgin and lactating mice are difficult to make. However, it may be assumed that if the contribution by caseins to the microsomal proteins could be discounted, the calculated specific activity of Δ5 desaturase in the IMFP of lactating mice is likely to be higher than the observed value. It should be noted here that protein content of CMFP did not increase during pregnancy and lactation.

The mammary tissue explants from lactating mice have been shown to be poor in β oxidation of fatty acids (48,49). In our experiments, the same has been found to be true with linoleate. However, the explants from IMFP of virgin mice can cause appreciable oxidation of 18:2ω6. As a result, a large percentage of saturated fatty acids in the virgin tissue becomes radiolabeled by [^{14}C]acetyl-CoA produced by oxidation of [1-^{14}C]18:2ω6. Therefore, it appears that the enzymatic activities in the differentiated tissues are regulated in favor of mobilization of stored linoleate, generation of 20:4ω6, and protection of available linoleate from oxidative degradation. These changes in linoleate metabolism seem to be compatible with the essential role of linoleate in mammary functions. Since all explants were cultured in the same hormone-supplemented medium, the differences in fatty acid metabolism observed in the mammary explants of virgin, midpregnant and lactating mice were not caused by the incubation conditions. Rather, they probably represent biochemical characteristics of the tissues concerned.

In conclusion, it appears that the hormonal conditions during pregnancy bring about striking differences in metabolic response between mammary fat cells and other fatty tissues. Lactation allows mammary tissues to preserve dietary linoleate (without much oxidative degradation), a precursor of eicosanoids which act like agonists for mammary functions.

ACKNOWLEDGMENT

This work was supported by grant CA 40160 awarded by the National Institutes of Health.

REFERENCES

1. Topper, Y.J., and Freeman, C.S. (1980) *Physiol. Rev. 60,* 1049–1106.
2. Imagawa, W., Bandyopadhyay, G.K., and Nandi, S. (1990) *Endocrinol. Rev. 11,* 49–523.
3. Vernon, R.G., and Flint, D.J. (1984) in *Physiological Strategies in Lactation* (Peaker, M., Vernon, R.G., and Knight, C.H., eds.) pp. 119–145, Academic Press, London.
4. Smith, G.H., Crabtree, B., and Smith, R.A. (1983) in *Biochemistry of Lactation* (Mepham, T.B., ed.) pp. 121–140, Elsevier, Amsterdam.
5. Dils, R.R. (1983) in *Biochemistry of Lactation* (Mepham, T.B., eds.) pp. 141–157, Elsevier, Amsterdam.
6. Mayer, R.J. (1978) *Vitamins and Hormones 36,* 101–163.
7. McNamara, J.P. (1991) *J. Dairy Sci.* 74, 706–719.
8. Williamson, D.H. (1980) *FEBS Lett. (Suppl.) 117,* K93–K105.
9. Ros, M., Lobato, M.J.F., Ruiz-Garcia, J.P., and Moreno, F.J. (1990) *Mol. Cell. Biochem. 93,* 185–194.
10. Dayton, S., Hashimoto, S., and Pearce, M.L. (1967) *J. Lipid Res. 8,* 508–510.
11. Body, D.R. (1988) *Prog. Lipid Res. 27,* 39–60.
12. van Staveren, W.A., Deurenberg, P., Katan, M.B., Burema, J., de Groot, L.C.P.G.M., and Hoffmans, M.D.A.F. (1986) *Am. J. Epidemiol. 123,* 455–463.
13. Knazek, R.A., and Liu, S.C. (1979) *Proc. Soc. Exp. Biol. Med. 162,* 346–350.
14. Knazek, R.A., Liu, S.C., Bodwin, J.S., and Vonderhaar, B.K. (1980) *J. Natl. Cancer Inst. 64,* 377–382.
15. Miyamoto-Tiaven, M.J., Hillyard, L.A., and Abraham, S. (1981) *J. Natl. Cancer Inst. 67,* 179–188.
16. Faulkin, L.J., Abraham, S., Mitchell, D.J., and Hillyard, L.A. (1986) *Proc. Soc. Exp. Biol. Med. 181,* 575–585.
17. Faulkin, L.J., and DeOme, K.B. (1960) *J. Natl. Cancer Inst. 24,* 953–959.
18. Hoshino, K. (1964) *Anat. Rec. 150,* 221–235.
19. Hoshino, K. (1978) in *Physiology of Mammary Glands* (Yokoyama, A., Mizuno, H., and Nagasawa, H., eds.) pp. 165–227, Japan Scientific Societies, Tokyo.
20. Shyamala, G., and Ferenczy, A. (1984) *Endocrinology 110,* 1078–1081.
21. Haslam, S.Z. (1988) *Endocrinology 122,* 860–867.
22. Haslam, S.Z., and Counterman, L.J. (1991) *Endocrinology 129,* 2017–2023.
23. McNarnara, J.P., Dehoff, M.H., and Collier, R.J. (1985) *J. Animal Sci. 61,* 410–415.
24. Blachier, F., Lacroix, M., Ahmud, A.M., Leger, C., and Ollivier-Bousquet, M. (1988) *Prostaglandins 35,* 259–276.
25. Bandyopadhyay, G., Imagawa, W., Wallace, D., and Nandi, S. (1987) *J. Biol. Chem. 262,* 2750–2756.
26. Bandyopadhyay, G., Imagawa, W., Wallace, D., and Nandi, S. (1988) *J. Biol. Chem. 263,* 7567–7573.
27. Levay-Young, B.K., Bandyopadhyay, G.K., and Nandi, S. (1987) *Proc. Natl. Acad. Sci. USA 84,* 8448–8452.
28. DeOme, K.B., Faulkin, L.J., Bern, H.A., and Blair, P.B. (1959) *Cancer Res. 19,* 515–520.
29. Topper, Y.J., Oka, T., and Vonderhaar, B.K. (1975) *Methods Enzymol. 39,* 443–454.
30. Bandyopadhyay, G.K., McKenzie, K.E., Imagawa, W., and Nandi, S. (1989) *Biochem. Biophys. Res. Commun. 158,* 730–736.
31. Dittmer, J.C., and Wells, M.A. (1969) *Methods Enzymol. 14,* 482–530.
32. Snyder, F., and Stephens, N. (1959) *Biochim. Biophys. Acta 34,* 244–245.
33. Ames, B.N. (1966) *Methods Enzymol. 8,* 115–118.
34. Mandon, E.C., de Gomez-Dumm, I.N.T., de Alaniz, M.J.T., Marra, C.A., and Brenner, R.R. (1984) *J. Lipid Res. 28,* 1377–1383.
35. Nicoll, C.S., and Tucker, H.A. (1965) *Life Sci. 4,* 993–1001.
36. Kuhn, N.J. (1977) *Symp. Zool. Soc. Lond. 41,* 16–192.
37. Ashby, J.P., Shirling, D., and Baird, J.D. (1982) *J. Endocrinol. 93,* 391–395.
38. Shirling, D., Ashby, J.P., and Baird, J.D. (1981) *J. Endocrinol. 90,* 285–294.
39. Knopp, R.H., Saudek, C.D., Arky, R.A., and O'Sullivan, J.B. (1973) *Endocrinology 92,* 984–988.
40. Flint, D.J., Sinnett-Smith, P.A., Clegg., R.A., and Vernon, R.G. (1979) *Biochem. J. 182,* 421–427.
41. Hamosh, M., Clary, T.R., Chernick, S.S., and Scow, R.O. (1970) *Biochim. Biophys. Acta 210,* 473–482.
42. Scow, R.O., and Chernick, S.S. (1987) in *Lipoprotein Lipase* (Borensztajn, J., ed.) pp. 149–186, Evener, Chicago.
43. Baldwin, R.L., and Yang, Y.T. (1974) in *Lactation: A Comprehensive Treatise* (Larson, B.L., and Smith, V.R., eds.) vol. 1, pp. 349–411, Academic Press, New York.

44. Vernon, R.G., and Flint, D.J. (1983) *Proc. Nutr. Soc. 42,* 315–331.
45. Camps, L., Reina, M., Llobera, M., Vilaro, S., and Olivecrona, T. (1990) *Am. J. Physiol. 258,* C673–C681.
46. de Gomez-Dumm, I.N.T., de Alaniz, M.J.T., and Brenner, R.R. (1983) *Lipids 18,* 781–788.
47. Brenner, R.R. (1981) *Prog. Lipid Res. 20,* 41–47.
48. Rao, G.A., and Abraham, S. (1975) *Lipids 7,* 409–412.
49. Robinson, A.M., and Williamson, D.H. (1978) *Biochem. J. 170,* 609–613.

[Received January 10, 1994, and in revised form December 19, 1994; Revision accepted December 28, 1994]

Dietary Oligofructose Lowers Triglycerides, Phospholipids and Cholesterol in Serum and Very Low Density Lipoproteins of Rats

Maria Fiordaliso[a], Nadine Kok[a], Jean-Pierre Desager[b], Fabienne Goethals[a], Dominique Deboyser[a], Marcel Roberfroid[a] and Nathalie Delzenne[a,*]

[a]Unité de Biochimie Toxicologique et Cancérologique, Département des Sciences Pharmaceutiques and [b]Laboratoire de Pharmacothérapie, Faculté de Médecine, Université Catholique de Louvain, B-1200 Brussels, Belgium

ABSTRACT: The present study was aimed at answering the question why feeding rats an oligofructose (OFS) supplemented diet could cause a significant reduction in plasma lipid levels. Daily administration of a 10% (w/w) OFS-containing diet to normolipidemic male rats resulted in a decrease in plasma triglycerides, phospholipids and cholesterol. The triglyceride-lowering effect was observed after one week and lasted for at least 16 wk and was associated with a reduction in plasma very low density lipoproteins, indicating that the hypolipidemic effect of OFS may be due to changes in liver lipid metabolism. We therefore tested whether OFS feeding modified the capacity of the liver to synthesize triglycerides from free fatty acids. Hepatocytes isolated from livers of control and OFS-fed rats were incubated in the presence of [1-^{14}C]palmitate, and both intracellular and extracellular [^{14}C]triglyceride formation were quantified. We found that chronic feeding of an OFS-supplemented diet to rats significantly reduced the capacity of isolated hepatocytes to synthesize triglycerides from palmitate. The results suggest that, like other soluble dietary fibers, OFS significantly alters liver lipid metabolism, resulting over time in a significant reduction in plasma triglyceride, phospholipid and cholesterol levels.

Lipids 30, 163–167 (1995).

Oligofructose (OFS) is an oligomer of ß-D-fructose containing two to nine monomeric units which are linked by ß (2-1) glycosidic bridges. OFS is resistant to hydrolysis by pancreatic amylase and saccharidases (1,2). OFS thus belongs to the class of nondigestible oligosaccharides (NDO) which share many properties with fermentable dietary fibers (3). Being nondigestible, OFS reaches the colon intact where it is fermented mostly by *Bifidobacterium* (4). This process produces energy for bacterial proliferation, short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, as well as lactate, which all feed the host. Mostly gaseous by-products (CO_2, CH_4 and H_2) are excreted (5).

In an earlier study, we had shown that feeding rats a diet containing 20% OFS for 30 d led to a significant decrease in the concentration of both serum and liver triglyceride levels (6). This suggested that the capacity of OFS-fed rats to esterify and secrete fatty acids as very low density lipoproteins (VLDL) may be reduced when compared to isocalorically fed control rats.

Similar effects of nondigestible, but fermentable, carbohydrates (including the synthetic fructooligosaccharides that are chemically similar to OFS) on serum triglycerides have been reported (7). It has been hypothesized that some SCFA, in particular propionate and possibly acetate, could be the mediators of this systemic modulation of the metabolism of triglycerides and of other lipids due to dietary fiber. However, the biochemical mechanism of this modulation remained largely undefined and may also vary for different food ingredients. Moreover, it may not be limited to the synthesis of triglycerides or the secretion of VLDL.

We have therefore followed the time course of the hypolipidemic effect of OFS under conditions that would exclude any changes in body weights. Furthermore, the lipid compositions of serum lipoproteins during OFS feeding were compared to those of control rats. Finally, an *ex vivo* protocol was developed to study the influence of OFS feeding on the capacity of hepatocytes isolated from these rats to incorporate [1-^{14}C]palmitate into triglycerides.

MATERIALS AND METHODS

Chemicals. OFS (purity ≥98%) was supplied as Raftilose®P95 by Raffinerie Tirlemontoise Inc. (Tienen, Belgium). It contains at least 95% (w/w) fructooligosaccharides with a mean degree of polymerization of 4.8. Collagenase and Dulbecco's medium were purchased from Boehringer-

*To whom correspondence should be addressed at Unité BCTC, UCL 7369, Avenue Mounier, 73 B-1200 Brussels, Belgium.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; HDL, high-density lipoprotein; IDL, intermediate density lipoprotein; LDL, low-density lipoprotein; NDO, nondigestible oligosaccharide; OFS, oligofructose; SCFA, short-chain fatty acids; SEM, standard error of the mean; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

Mannheim (Mannheim, Germany) and Flow Laboratories (Irvine, Scotland), respectively. Bovine serum albumin (BSA) (fraction V) was from the Sigma Chemical Company (St Louis, MO); [1-^{14}C]palmitic acid (710 μCi/mmol) was from New England Nuclear Research Products (Boston, MA); Carbo Sorb and Permafluor scintillation mixtures were from Beckman (Los Angeles, CA). All other chemicals were of the purest grade available.

Animals and diets. Male Wistar rats ICO-WY IOPS from Iffa Credo (Les Oncins, France) initially weighing about 100 g were housed individually in grill-bottom metal wire cages in a room maintained under a 12 h light/dark cycle at 21 ± 3°C. The animals were acclimatized for one week prior to the experiment. All animals had free access to food and water.

The powdered basal diet A04 was purchased from UAR (Villemoisson-Sur-Orge, France). It contained 58% (w/w) carbohydrates, including starch 33% (w/w) and fiber (3% w/w), 17% (w/w) proteins, 12% (w/w) water, 4% (w/w) cellulose, 3% (w/w) lipids and 5% (w/w) minerals. Vitamin A and D3 levels were 7500 and 1500 UI/kg, respectively.

The diets used for the experiments were: (i) the control diet, corresponding to the A04 diet (caloric value, 2.9 Kcal/g); (ii) the sucrose-supplemented diet (5% wt/ wt) prepared by adding 5 g of dry saccharose to 95 g of AO4 diet (caloric value, 2.9 Kcal/g); (iii) the OFS-enriched diet (10% w/w) prepared by adding 10 g of dry OFS to 90 g of AO4 diet (caloric value, 2.8 Kcal/g, assuming that the OFS caloric value is 2 Kcal/g; Ref. 8).

Animal experiments were performed consistent with the rules for humane care according to Belgian State Directives.

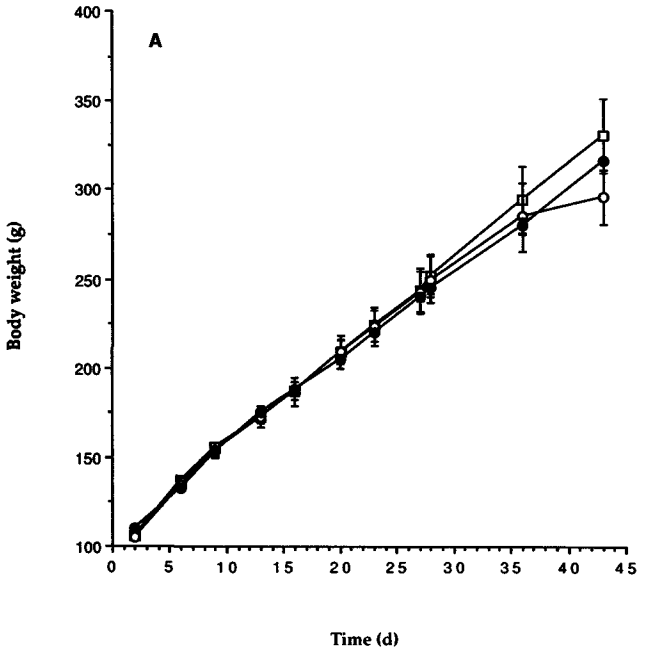
Experimental protocols. Experiment 1 compared food intake and body weight of rats fed control and OFS-containing (10% w/w) diets. However, as the OFS supplement not only diluted the diet but also slightly modified its caloric content, a diet was prepared which was supplemented with 5% w/w sucrose. This assumed that OFS had ≤50% the caloric content of sucrose (8). Following the acclimatization period, 15 rats were weight matched and assigned to three groups which received one of the three diets for up to 7 wk. Body weight, 24-h food intake and fecal excretion were measured twice weekly. The feces collected on week 6 were frozen, lyophilized and then used for lipid analysis. Experiment 2 used two groups of ten rats and was designed to monitor the time course (up to 12 wk) of the effect of OFS feeding on serum triglycerides, which was measured once every week and then at the end of the treatment (week 16). Experiment 3 was the *ex vivo* protocol in which two groups of ten rats fed either a control or an OFS-containing (10% w/w) diet for 5–6 wk were used to prepare hepatocytes according to the following procedure: after feeding, rats were anesthetized at 10:00 a.m.; the liver was excised; and hepatocytes were isolated by the collagenase perfusion technique of Berry and Friend (9) as adapted by Krack *et al.* (10). Hepatocytes (≥90% viability as measured by Trypan blue exclusion) were then incubated at a final concentration of 0.5 10^6 cells/mL in Dulbecco's medium containing 5×10^{-5}M [1-^{14}C]palmitate (0.7 mCi/mmol) bound to BSA and prepared according to Capuzzi *et al.* (11). The reaction was carried out for 150 min in an incubation vessel with a rotating helix (40 rpm) as previously described (12). At 30-min intervals, samples of the suspension were removed for biochemical and radiochemical analysis. Each assay was carried out in duplicate, and at least eight separate experiments were performed.

Analytical procedures. For the enzymatic measurement of serum triglycerides, post-prandial blood samples were collected through the caudal vein. For the separation of lipoproteins, blood was collected from the vena cava of rats that had been fasted for 18 h. Serum was then ultracentrifuged on a density gradient (rotor SW41, 40 000 rpm, 18 h) according to Chapman *et al.* (13). Triglycerides, phospholipids and total and esterified cholesterol were assayed in total serum and in each lipoprotein fraction using Boehringer kits. The feces collected on week 6 in protocol 2 were frozen, then dried and powdered. Total lipids were extracted with chloroform/methanol 1:1. The organic phase was evaporated under nitrogen, and the residue was solved in ethanol. Total (free and esterified) fatty acids were measured using a kit from Boehringer-Mannheim. The synthesis of triglycerides and secretion of lipoproteins by isolated hepatocytes were assayed radiochemically. Both the incorporation of [1-^{14}C]palmitate into cellular triglycerides and the increase in [^{14}C]lipids in the culture medium were measured as described by Deboyser *et al.* (12). The activity of palmitoyl coenzyme A synthetase in isolated hepatocytes was measured by the spectrophotometric method of Ichara and Shibasaki (14). All results of the *ex vivo* experiment were expressed per mg of cellular protein which was determined by the method of Lowry *et al.* (15).

Statistical analysis. Data are expressed as mean ± standard error (SEM). To compare the time course of the effect of OFS feeding on body weight and food intake (Experiment 1) or the *in vitro* incubation of hepatocytes on [1-^{14}C]palmitate incorporation (Experiment 3), analysis of variance (ANOVA) using a two-way ANOVA program was performed, followed by a Sheffe test to analyze time/treatment interaction on food intake. Statview^{TM} 512^{+} software was used. To compare the lipid composition of lipoproteins and the concentration of serum triglycerides in Experiment 2, Student's *t*-tests were performed. In all statistical analyses, $P < 0.05$ was used as the significance level to analyze OFS effects.

RESULTS

Body weight growth and food intake (Experiment 1). The rats fed the diet supplemented with OFS gained weight each day at a similar rate (variance analysis; $P > 0.05$) as did the controls and sucrose-supplement fed rats (Fig. 1A). Food consumption was not significantly different (variance analysis; $P > 0.05$) between experimental groups (Fig. 1B), despite a tendency for OFS-fed rats to eat more than did the rats of the other groups. All animals fed OFS over time appeared healthy and behaved normally.



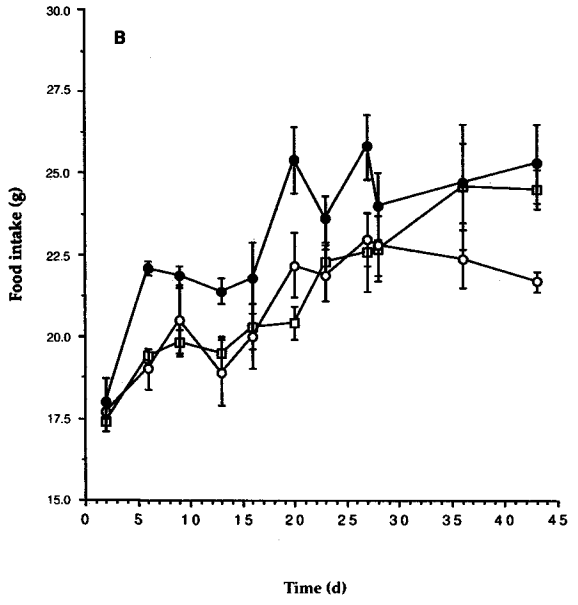


FIG. 1. Body weight (A) and food intake (B) of rats fed a control diet (□) or a diet supplemented with either 5% w/w sucrose (○) or 10% w/w oligofructose (OFS) (●). The values represent means ± SEM of five rats in each group. Variance analysis based on a two-way analysis of variance was used to test for time/treatment interaction. No statistically significant effect ($P > 0.05$) of OFS feeding was apparent.

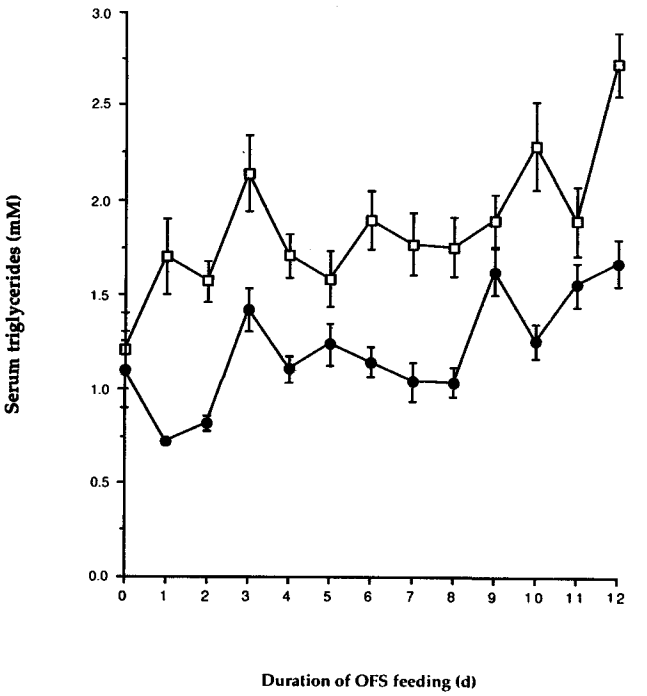


FIG. 2. Time course of serum triglycerides (mmol/L) of rats fed a standard (□) or an oligofructose (OFS)-supplemented (●) diet. The values represent means ± SEM of five animals in each experimental group. Variance analysis based on a two-way analysis of variance was used to test for time/treatment interaction. It showed a statistically significant effect ($P < 0.05$) of OFS feeding.

Serum lipids and lipoproteins (Experiment 2). Chronic administration of OFS to the rats caused a significant ($P < 0.05$) reduction in serum triglyceride levels (Fig. 2). The triglyceride-lowering effect was significant ($P < 0.05$) after one week of feeding and lasted for up to 16 wk ($P < 0.05$). Such a decrease in serum triglycerides was unlikely to be due to changes in fecal excretion of lipids. Indeed, fecal analysis at week 6 showed no statistically significant differences in 24-h excretion of esterified fatty acids between standard and OFS-supplement fed rats (15.8 ± 2.4 μmol vs. 14.9 ± 1.1 μmol esterified fatty acids for control and treated rats, respectively). Table 1 shows that OFS-feeding for 16 wk caused a significant ($P < 0.05$) reduction in serum triglycerides (25% decrease), phospholipids (15% decrease) and total cholesterol (15% decrease). This was exclusively due to the reduction of lipids in the VLDL fraction with the composition of both LDL and HDL remaining unchanged. Moreover, the effect of OFS on VLDL was likely to be due to a reduction in the number rather than a change in the composition of VLDL particles. Indeed, neither the composition nor the size estimated [according to Fungwee *et al.* (16)], by calculating the ratio of the sum of phospholipids plus free cholesterol over the serum triglycerides and esterified cholesterol, showed a statistically significant difference (Table 2).

Effect of OFS on the synthesis and secretion of triglycerides in isolated hepatocytes (Experiment 2). The incorporation of [1-^{14}C]palmitate into cellular triglycerides, as a function of incubation time, in hepatocytes isolated from OFS-fed rats was lower than in control cells ($P < 0.05$) (Fig. 3A). After 150 min of incubation, the incorporation of [1-^{14}C]palmitate into cellular triglycerides was reduced by 40% ($P < 0.05$). Apart from this reduction in the cellular incorporation of [1-^{14}C]palmitate, the accumulation over time of [^{14}C]triglyc-

TABLE 1
Effects of Feeding Rats an Oligofructose-Containing Diet (10%) on Various Lipid Parameters of Serum[a]

	Concentrations (mM) ± SEM (n)				
	Triglycerides	Phospholipids	Total cholesterol	Esterified cholesterol	Free cholesterol
Serum control	1.14 ± 0.10 (10)	1.38 ± 0.03 (10)	1.70 ± 0.08 (10)	1.28 ± 0.10 (7)	0.46 ± 0.04 (7)
OFS	0.87 ± 0.08 (10)[b]	1.17 ± 0.04 (010)[b]	1.45 ± 0.05 (10)[b]	1.04 ± 0.04 (7)[b]	0.45 ± 0.03 (7)
VLDL Control	0.79 ± 0.01 (10)	0.16 ± 0.02 (9)	0.15 ± 0.02 (10)	0.06 ± 0.006 (10)	0.10 ± 0.01 (10)
OFS	0.48 ± 0.07 (8)[b]	0.10 ± 0.01 (9)[b]	0.11 ± 0.01 (9)[b]	0.05 ± 0.052 (9)	0.07 ± 0.01 (10)
LDL Control	0.06 ± 0.01 (10)	0.28 ± 0.02 (9)	0.54 ± 0.04 (10)	0.35 ± 0.03 (7)	0.13 ± 0.02 (7)
OFS	0.09 ± 0.01 (9)[b]	0.26 ± 0.02 (9)	0.51 ± 0.03 (9)	0.37 ± 0.02 (9)	0.14 ± 0.01 (9)
HDL Control	0.08 ± 0.005 (10)	0.52 ± 0.03 (10)	0.84 ± 0.07 (10)	0.73 ± 0.07 (10)	0.12 ± 0.01 (10)
OFS	0.08 ± 0.005 (8)	0.45 ± 0.03 (8)	0.81 ± 0.07 (7)	0.69 ± 0.06 (7)	0.12 ± 0.01 (9)

[a]Values represent means ± SEM; numbers in parentheses are the number of samples analyzed, each sample being from one rat. Abbreviations: OFS, oligofructose; VLDL, very low density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; n is the number of samples.
[b]$P < 0.05$ for difference between control and treated rats.

TABLE 2
Composition and Size of Serum VLDL Particles in Rats Fed Standard or OFS-Supplemented Diets[a]

	Experimental group	
VLDL	Controls (n = 9)	OFS (n = 8)
	(composition%)	
Triglycerides	68 ± 1	65 ± 1
Phospholipids	12 ± 0.1	13 ± 0.4
Total cholesterol	6 ± 0.3	7 ± 0.4
Total free cholesterol	3.7 ± 0.4	3.3 ± 0.2
Protein	13 ± 0.4	15 ± 1
Size (calculated)	0.30 ± 0.03	0.30 ± 0.04

[a]Values represent means ± SEM; n is the number of samples analyzed. Student's *t*-test was used to compare the experimental data, which showed no statistical difference. See Table 1 for abbreviations.

erides in the incubation medium was not statistically different ($P > 0.05$) under the two experimental conditions (Fig. 3B).

Palmitoyl-CoA synthetase activity. The rate of activation to acyl-CoA is limiting in the incorporation of fatty acids into cellular triglycerides (17). As adaptive changes in the activity of the enzyme involved in this activation may reflect alterations in the metabolic flux, we examined whether palmitoyl-CoA synthetase activity was altered after OFS feeding. However, the activity was not statistically different ($P > 0.05$) in homogenates of hepatocytes isolated from controls and OFS-fed rats (2.5 ± 0.2 and 2.5 ± 0.1 mmol/min/mg protein), respectively.

DISCUSSION

Our data show that feeding rats a diet supplemented with 10% OFS had no significant effect on body weight gain. Besides a slight but not significant increase in macro- and micronutrient intake, the chronic feeding of OFS significantly reduced serum triglycerides when measured in both fed and fasted rats. Moreover, in the fasted state, serum phospholipids and total and esterified cholesterol were also significantly reduced. These data confirm and extend our previous observations (6) and the findings of others (7) that the OFS-like synthetic fructo-oligosaccharides called Neosugar®, similarly reduce triglyceridemia by 30% after feeding to rats for a 6-wk period of a supplemented (10 to 20% w/w) diet. Our data also agree with previous observations that fermentable dietary fibers, such as pectins, are effective nondigestible carbohydrates that reduce serum and liver triglycerides (18,19).

Using an *ex vivo* protocol, we have also shown that OFS feeding results in a reduced capacity of isolated hepatocytes to incorporate free fatty acids into cellular triglycerides. Within the period of observation, no effect on secretion of newly synthesized triglycerides occurred.

The observation that reduced serum triglyceride, phospholipid and cholesterol levels were mainly due to a decrease in the number of VLDL particles, together with the results of our *ex vivo* experiments, supports the hypothesis that OFS feeding, like that of other fermentable dietary fibers (20), significantly alters the metabolism of lipids in the liver with, as a likely consequence, a reduction in VLDL production. Such an effect has also been demonstrated for lovastatin treatment (21). However, an increase in the catabolism of VLDL by lipoprotein lipase, as observed with fibrates, cannot, as yet, be discounted.

The observation that hepatocytes isolated from OFS-fed rats had a lower capacity than those from control rats to incorporate [1-^{14}C]palmitate into cellular triglycerides still needs to be explained. Since the activity of palmitoyl-CoA synthetase was not changed, we postulate that the availability of acyl-CoA is modified in the hepatocytes of OFS-fed rats. Two possibilities could be explored: either the pattern of esterification of acyl-CoA could be altered or the catabolism of acyl-CoA to ketone bodies could be stimulated. In the latter case, regulation of the key enzyme carnitine acyltransferase would likely be involved. Whatever metabolic change is responsible for the hypolipidemic effect, however, one must still explain how a nondigestible carbohydrate can regulate systemic lipid metabolism if, as shown in this study, it has no significant effect on the fecal excretion of lipids. The most likely mediators of such an effect may be the SCFA and/or lactate which are produced in significant amounts by fermenting bacteria. These carboxylic acids are absorbed through the intestinal membrane and reach the liver. Moreover, they have already been shown to act as regulators of key hepatic meta-

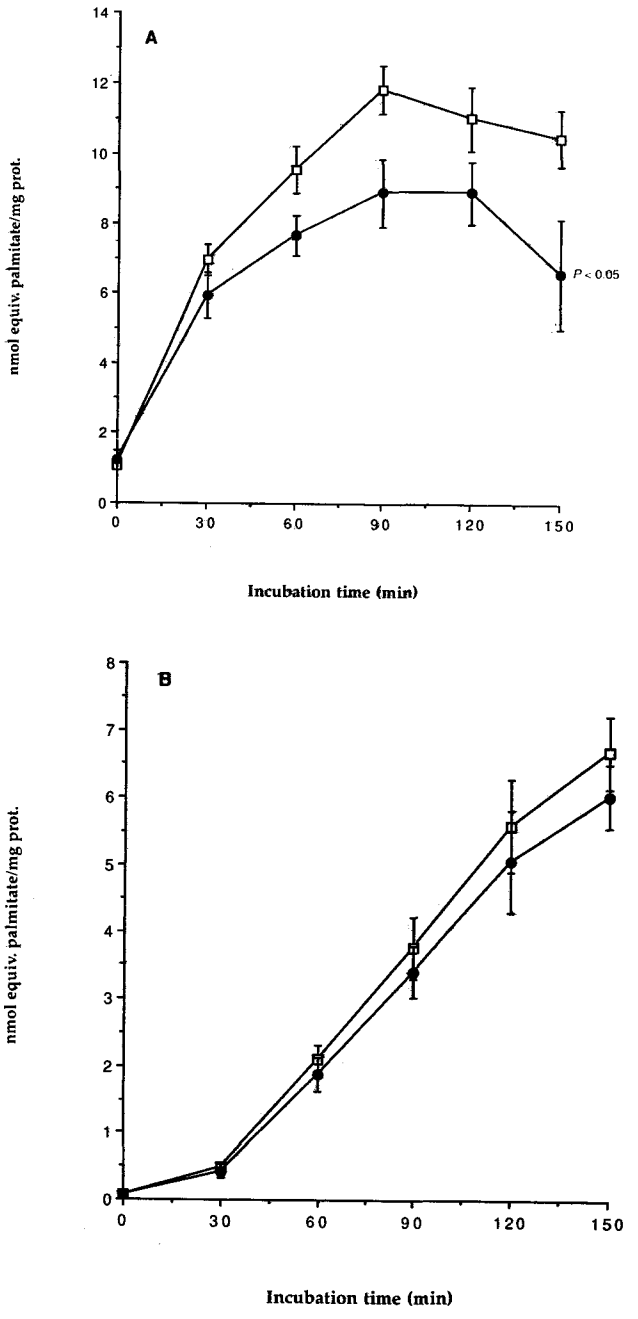


FIG. 3. Effect of oligofructose (OFS) feeding on the capacity of isolated hepatocytes to incorporate [1-^{14}C]palmitate into cellular triglycerides (A) and to secrete [^{14}C] very low density lipoprotein (VLDL) into the culture medium (B). Hepatocytes from control (□) or OFS-fed rats (●) were incubated for 150 min in Dulbecco's medium and analyzed as described in the Materials and Methods section. Variance analysis based on a two-way analysis of variance was used to test time/treatment interaction which showed statistically significant changes ($P < 0.05$) for OFS feeding for incorporation into cellular triglycerides (A) but not for VLDL release (B).

bolic pathways (22). Studies are in progress to test these hypotheses and, in particular, the role of the liver in mediating the hypolipidemic effect.

ACKNOWLEDGMENTS

We gratefully acknowledge the Fellowships received from the Belgian Institute for Applied and Agricultural Research (IRSIA) (Maria Fiordaliso), and the Fund for Scientific Development, Université Catholique de Louvain, Belgium (FDS-UCL) (Nadine Kok and Nathalie Delzenne).

REFERENCES

1. Oku, T., Tokunaga, T., and Hosoya, N. (1984) *J. Nutr. 114*, 1574–1581.
2. Rumessen, J.J., Bode, S., Hamberg, O., and Hoyer, E.G. (1990) *Am. J. Clin. Nutr. 52*, 675–681.
3. Roberfroid, M. (1993) *Critical Reviews in Food Science and Nutrition 33*, 103–148.
4. Modler, H.W., McKellar, R.C., and Yaguchi, M. (1990) *J. Inst. Can. Sci. Technol. Aliment . 23*, 29–41.
5. Wang, X., and Gibson, G.R. (1993) *Appl. Bacteriol. 75*, 373–380.
6. Delzenne, N., Kok, N., Fiordaliso, M., Deboyser, D., Goethals, F., and Roberfroid, M. (1993) *Am. J. Clin. Nutr. (suppl.) 57*, 820S.
7. Tokunaga, T., Oku, T., and Hosoya, N. (1986) *J. Nutr. Sci. Vitaminol. 32*, 111–121.
8. Roberfroid, M., Gibson, G., and Delzenne, N. (1993) *Nutr. Rev. 51*, 137–146
9. Berry, M.N., and Friend, D.S. (1969 *J. Cell Biol. 43*, 506–520.
10. Krack, G., Gravier, O., Roberfroid, M., and Mercier, M. (1980) *Biochim. Biophys. Acta 632*, 619–629.
11. Capuzzi, D.M., Kackman, R.D., Uberti, M.M., and Ree, M.A. (1974) *Biochem. Biophys. Res. Common. 60*, 1499–1508.
12. Deboyser, D., Goethals, F., Krack, G., and Roberfroid, M. (1989) *Toxicol. Appl. Pharmacol. 97*, 473–479.
13. Chapman, M.J., Goldstein, S., Lagrangeand, D., and Laplaud, M.P. (1981) *J. Lipid Res. 22*, 339–358.
14. Ichara, K., and Shibasaki, Y. (1991) *J. Lipid Res. 32*, 1709–1712.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265–273.
16. Fungwee, T.V., Cagen, L., Wilcox, H.G., and Heimberg, M. (1992) *J. Lipid Res. 33*, 179–191.
17. Groot, P.H.E., Scholte, H.R., and Hülsmann, W.C. (1976) *Adv. Lipid Res. 14*, 75–126.
18. Tsuji, Y., Yamamada, K., Hosoya, N., and Moriuchi, S. (1986) *J. Nutr. Sci. Vitaminol. 32*, 93–100.
19. Vigne, J.L., Lairon, D., Borel, P., Portugal, H., Pauli, A.M., Hauton, J.C., and Lafont, H. (1987) *Br. J. Nutr. 58*, 405–413.
20. Mazur, A., Gueux, E., Felgines, C., Bayle, D., Nassir, F., Demigné, C., and Rémésy, C. (1992) *Proc. Soc. Exp. Biol. Med. 199*, 345–350.
21. Kasim, S.E., Leboeuf, R.C., Khilnani, S., Tallapaka, L., Dewunda, D., and Jen, D K.L.C. (1992) *J. Lipid Res. 33*, 1–7.
22. Demigné, C., Yacab, C., and Rémésy, C. (1986) *J. Nutr. 116*, 77–86.

[Received December 8, 1993, and in revised form October 31, 1994; Revision accepted December 20, 1994]

1-*O*-Alkyl-2,3-diacylglycerols in the Skin Surface Lipids of the Hairless Mouse

Hirosuke Oku[a,*], Jyutaro Shudo[a], Kunio Mimura[b], Akinori Haratake[b], Junichi Nagata[a] and Isao Chinen[a]

[a]Laboratory of Applied Biochemistry, Faculty of Agriculture, University of The Ryukyus, Nishihara-Cho, Okinawa-Ken 903-01 and [b]Kanebo Ltd., Cosmetic Laboratory, Kotobuki-Cho, Odawara-Shi, Kanagawa-Ken, Japan

ABSTRACT: A neutral lipid class was isolated by thin-layer chromatography from the skin surface lipids of the hairless mouse. The fraction migrated faster than triglycerides and had a migration rate similar to that of diacyl alkanediols (diester wax). Upon deacylation, however, the long-chain diols were identified as 1-alkylglycerol ethers based on their chromatographic properties and on the mass spectra of their nicotinylidene derivatives. Thus, the skin lipid fraction was identified as 1-*O*-alkyldiacylglycerol. The alkyl moieties were all saturated and even-numbered and ranged in chainlength from C_{16} to C_{22} with 1-*O*-hexadecylglycerol amounting to 34% of the total glycerol ether moieties. The fatty acids derived from this lipid fraction were mostly monoenoic with chainlengths ranging from C_{16} to C_{24}. The major acyl component was eicosenoic acid (20:1) representing 61% of the total fatty acids.
Lipids 30, 169–172 (1995).

A number of studies have been undertaken to characterize the skin surface lipids of mammalian species (1–6). In the course of our study on the lipid and fatty acid compositions of the skin surface lipids of the hairless mouse, a neutral lipid fraction was isolated by thin-layer chromatography (TLC). The neutral lipid migrated closely with diacylated glycerol ethers and showed a somewhat lower mobility than diester wax.

Downing and Sharaf (6) had described the presence of alkyldiacylglycerols in the skin surface lipid of guinea pig. On the basis of its migration rate on TLC, the unidentified lipid we isolated from the skin surface of the hairless mouse appeared to be similar to alkyldiacylglycerol. The present study was undertaken to confirm the presence of these neutral glycerol ether lipids in the skin surface of the hairless mouse.

MATERIALS AND METHODS

Chemicals. 1-*O*-Hexadecyl-2,3-dipalmitoyl-*rac*-glycerol and 1,2-di-*O*-hexadecyl-3-palmitoyl-*rac*-glycerol were purchased from Sigma Chemical Company (St. Louis, MO); 3-pyridine carboxaldehyde was from Aldrich Chemical Company (Milwaukee, WI). Other reagents used were all guaranteed grade and were purchased from Nacalai Tesque (Kyoto, Japan).

Extraction of the skin surface lipids of the mouse. Hairless mice (21–45 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and housed in stainless-steel cages at 25 ± 2°C under a 12-h light/dark cycle with light from 0800 to 2000 h. The animals were anesthetized with diethyl ether, and the skin surface lipids were extracted as described previously in detail (7). Briefly, reagent grade ethanol (20 mL) was poured over the back of the animals in such a manner that as little solvent as possible flowed over the ears, mouth and anal regions. The back was then repeatedly rinsed with ethanol which was collected in Petri dishes. After removing hair and other debris by filtration, the extract was brought to dryness on a rotary evaporator, redissolved in hexane, and stored at –20°C.

TLC. For the preparation of alkyldiacylglycerol, the skin surface lipids were applied to precoated silica gel plates (Merck, Darmstadt, Germany), and the plates were developed successively with hexane, benzene and hexane/diethyl ether/acetic acid (70:30:1, by vol). Lipid fractions were extracted from the silica gel with chloroform/methanol (2:1, vol/vol).

For analytical purposes, high-performance thin-layer chromatography (HPTLC) plates (Merck) were used. Chromatograms were sprayed with 50% sulfuric acid and were visualized by charring at 120°C for 15 min.

Gas chromatography/mass spectrometry (GC/MS). Alkyldiacylglycerols isolated by TLC were hydrolyzed in ethanolic alkali (0.5 N KOH in 94% ethanol) at 40°C for 60 min. The fatty acids derived were methylated by heating with 5% anhydrous hydrogen chloride in methanol. The fatty acid methyl esters (5 to 10 μg) were analyzed on a gas chromatograph (GC 14A; Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector. A fused silica capillary column (CBP1-M50-025, 0.2 mm inner diameter × 50 m length; Shimadzu) was used with nitrogen serving as carrier gas at a flow rate of 0.35 mL/min. The column temperature was programmed from a hold at 50°C for 1 min, to 100°C at a rate of 10°C/min, and then further raised to the final temperature of 270°C at a rate of 5°C/min.

The fatty acids derived from alkyldiacylglycerols were also characterized by MS of their picolinyl esters. Picolinyl esters

*To whom correspondence should be addressed.

Abbreviations: GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography.

Copyright © 1995 by AOCS Press

of fatty acids were prepared as described previously (8). Fatty acids (200 μg) were dissolved in 0.1 mL of thionyl chloride and left to stand at room temperature for 1 min. The reagent was then evaporated to dryness under a nitrogen stream. A dilute solution of pyridyl carbinol (0.1 mL,1%) in acetonitrile was added, and the mixture was allowed to stand for 1 min. The mixture was then brought to dryness, and the reaction products were extracted into 20 μL of ethyl acetate. Aliquots of this extract were injected into the gas chromatograph.

The deacylation products obtained from alkyldiacylglycerols were converted into their nicotinylidene derivatives as described previously (9,10). For the present purpose, the non-saponifiable lipid extract containing alkylglycerol (about 300 μg) was evaporated to dryness. The sample together with 0.2 mL of *p*-toluenesulfonic acid (2 mg/mL acetonitrile) and 0.3 mL of freshly prepared pyridine-3-carboxyaldehyde (4 mg/mL acetonitrile) was kept at 90°C overnight, cooled, and diluted with 1 mL of water. The products were extracted with 1 mL ethyl acetate, the extract washed successively with water (1 × 1 mL) and saturated aqueous sodium chloride solution (2 × 1 mL), and dried over anhydrous sodium sulfate.

Both picolinyl and nicotinylidene derivatives were then analyzed by GC/MS. The mass spectrometer used was a Shimadzu GC/MS-QP1000EX. The column used was the same as described above and was operated with helium as carrier gas at a flow rate of 0.38 mL/min. The column temperature was programmed from a hold at 50°C for 1 min, to 100°C at a rate of 10°C/min, and then further raised to the final temperature of 300°C at a rate of 5°C/min. Ionization of samples was by electron impact at 70 eV.

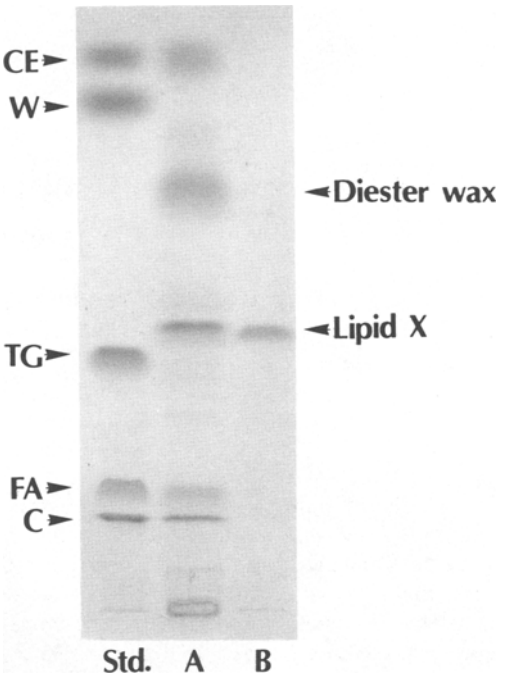


FIG. 1. High-performance thin-layer chromatography chromatogram of the skin surface lipids of the hairless mouse (lane A), 1-*O*-hexadecyl-2,3-dipalmitoyl-*rac*-glycerol (lane B), and of standards (Std.). The plates were developed successively with hexane, then with benzene to the top, and finally to half plate height with hexane/diethyl ether/acetic acid (70:30:1, by vol). The chromatogram was sprayed with 50% sulfuric acid and charred at 120°C for 15 min. Standards used were C, cholesterol; FA, fatty acid (oleic acid); TG, triglyceride (triolein); W, wax (stearyl oleate); CE, cholesteryl ester (cholesteryl oleate).

RESULTS

Figure 1 shows the HPTLC pattern of the skin surface lipids of the hairless mouse (lane A) together with alkyldiacylglycerol (lane B) and standards (Std.). A lipid fraction is seen in the region where alkylglycerides migrate, and it was tentatively named lipid X. This lipid accounted for about 15% of the total skin surface lipids of the hairless mouse as determined densitometrically.

Figure 2 shows the gas chromatogram of the nicotinylidene derivatives of the diols derived from lipid X (A) and diester wax (B). Consistent with previous observations, the nicotinylidene derivatives of alkanediol and monoalkylglycerols exhibited pairs of peaks due to the presence of diastereomers (*cis*- and *trans*-form, see Ref. 9). The isomer eluting earlier would be expected to be the *cis*-form due to its more compact nature. The nicotinylidene derivatives of the diols derived from lipid X were clearly distinguishable from those derived from diester wax. The peaks shown in the figure were identified by comparison of their retention times with those of authentic standards and based on their mass spectra as discussed in the following paragraphs.

Figure 3 shows the mass spectrum of fraction a1 (see Fig. 2A) in panel A and of the nicotinylidene derivative of 1-*O*-hexadecylglycerol in panel B, confirming the identity of the two compounds. The diagnostic ions of ether compounds are the fragment ions X, Y and Z. The formation of these fragments is illustrated in Figure 4A. The mass spectra of fractions b and c in Figure 2 showed similar fragmentation patterns and also exhibited the abundant diagnostic ions X, Y and Z. Thus, the peaks a–c were identified as the nicotinylidene derivatives of monoalkylglycerols with alkyl chains of 16, 18 and 20 carbons long.

Ions separated by 14 mass units are seen in the region between the molecular ion (M^+, m/z = 405) and the ion at m/z = 194. The ion at $[M - 15]^+$ had been of minimal intensity in the previous study (9) and was not seen in the present study. The mass spectrum shown in Figure 3A thus reflects the straight chain structure of 1-hexadecylglycerol. The base peak was the fragment at m/z = 57. The ratios of the intensities for the fragment X, Y and Z to that of peak m/z = 108 were 0.52, 0.20 and 0.44, respectively.

The mass spectra of peak c1 in Figure 2B and of the nicotinylidene derivative of hexadecane-1,2-diol are given in Figures 5A and B, respectively. The fragmentation pattern of peak c1 was quite similar to that of the nicotinylidene derivative of hexadecane-1,2-diol except for the presence of parent ion M^+ at m/z = 403. Peak c1 was therefore identified as the nicotinylidene derivatives of eicosane-1,2-diol (M^+, m/z = 347). A mechanism for the formation of these ions is proposed

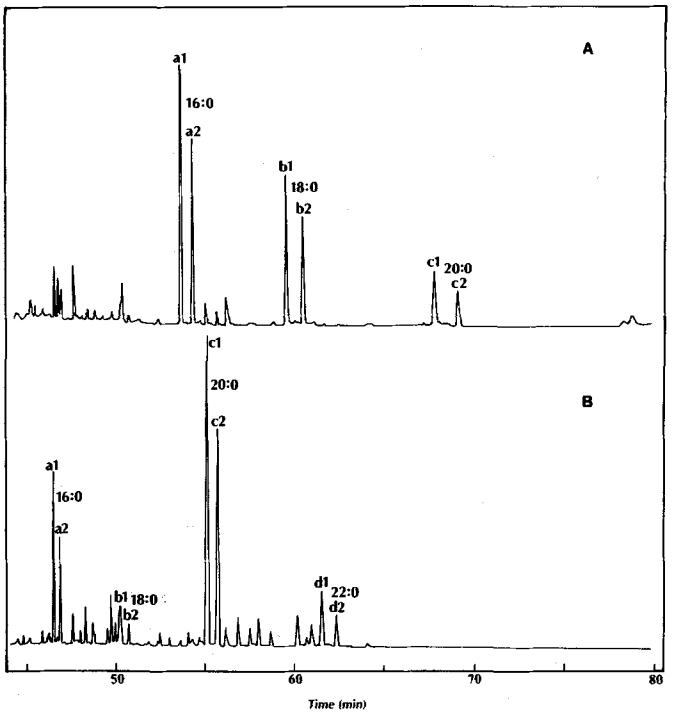


FIG. 2. Gas chromatograms of the nicotinylidene derivatives of the deacylated products derived from lipid X (A) and diester wax (B). The chainlengths for the alkyl ether group (A) and the diol (B) were determined by gas chromatography/mass spectrometry. Fractions a, b and c in panel A are the nicotinylidene derivatives of alkylglycerols with alkyl chainlengths of 16, 18 and 20 carbon atoms, respectively. Fractions a, b, c and d in panel B are the nicotinylidene derivatives of alkanediols with chainlength of 16, 18, 20 and 22 carbon atoms, respectively.

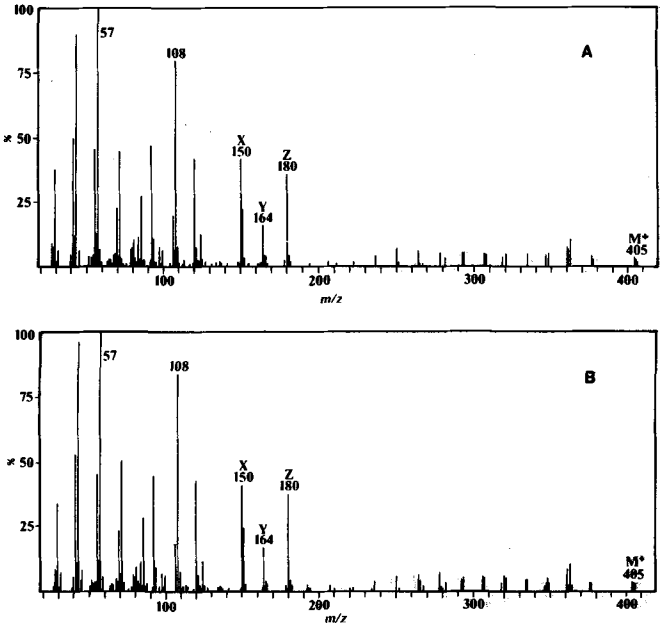


FIG. 3. Mass spectra of fraction a1 in Figure 2A (A) and the nicotinylidene derivative of 1-hexadecylglycerol (B). The ordinate is the abundance relative to the base peak (m/z = 57).

in Figure 4B. In this case, the fragment Z (m/z = 180) was not detectable. The relative intensity to that of the base peak m/z = 108 was 0.1 for fragment X (m/z = 150) and 0.02 for fragment Y (m/z = 164). The spectrum thus did not contain the diagnostic ions for alkylglycerol. As was the case for alkylglycerol, no evidence for the presence of methyl substitution in *iso*-position was apparent from the spectrum. A series of straight-chain alkane-1,2-diols with chainlengths of C_{16}–C_{22} were identified, as is shown in Figure 2B.

Table 1 lists the alkylglycerol and fatty acid composition of lipid X which is now identified as a mixture of monoalkyldiacylglycerols. The *O*-alkyl moieties of this lipid were mainly saturated, straight chains of C_{16}–C_{22}. The picolinyl esters of the fatty acids were analyzed by GC/MS and identified as shown in Table 1. The monoalkyldiacylglycerols contained fatty acids with chainlengths of C_{16}–C_{24}. The sum of the monoenoic fatty acids was about 73%, with 61% consisting of 20:1. Appreciable amounts of *iso*-fatty acids were present whereas only traces of *anteiso*-fatty acids were detectable.

Table 2 presents the diol and fatty acid composition of the diester wax. All the alkanediols identified had saturated and straight carbon chain structures with chainlengths of C_{16}–C_{22}. The most prominent component was eicosane-1,2-diol amounting to 48% of total alkanediols. The fatty acids of the diester wax showed less unsaturation than those of the alkyldiacylglycerols. The sum of monoenoic fatty acids was about 38%. In contrast to the alkyldiacylglycerols, the diol diester wax contained branched-chain fatty acids in significant proportions. The *iso*-series of branched-chain fatty acids (20%) predominated over the *anteiso*-series (2%) in this lipid class.

DISCUSSION

The presence of monoalkyldiacylglycerol has been shown here in the skin surface lipids of the hairless mouse for the

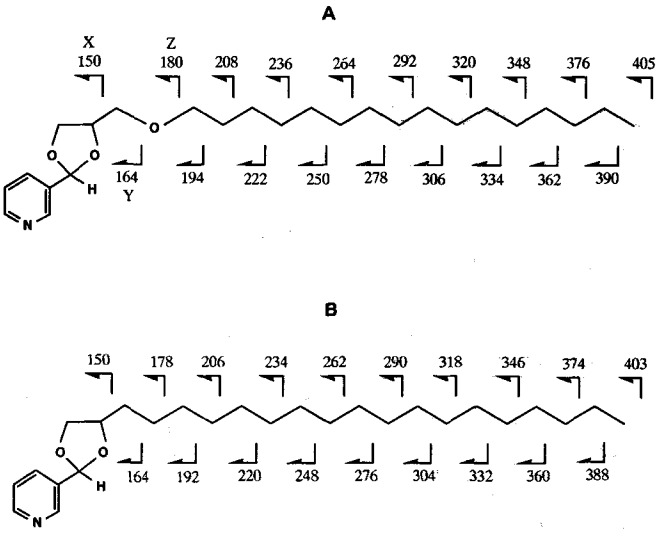


FIG. 4. Fragmentation of nicotinylidene derivative of 1-hexadecylglycerol (A) and eicosane-1,2-diol (B). The numbers above the arrow show the m/z of ions generated by cleavage at the site indicated.

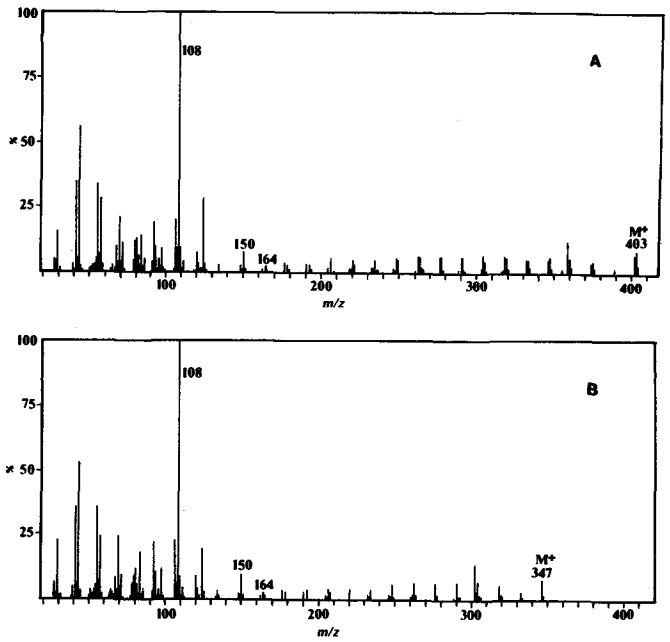


FIG. 5. Mass spectra of fraction c1 in Figure 2B (A) and the nicotinylidene derivative of hexadecane-1,2-diol (B). The ordinate is the abundance relative to the base peak (*m/z* = 108).

TABLE 1
Long-Chain Alkyl and Fatty Acid Compositions of Monoalkyldiacylglycerols from Skin Surface Lipids of the Hairless Mouse[a]

Carbon number	Alkyl	Fatty acid		
		Straight		
	Straight	Saturated	Monoenoic	Branched-*iso*
15				
16	34.3	0.8	0.2	0.4
17				
18	27.7	2.1	2.0	0.7
19		0.4		0.1
20	12.5	13.0	60.6	1.2
21		0.5		0.2
22	1.3	4.0	8.7	0.3
23		0.1		0.1
24		1.6	1.3	0.5
Sum	75.8	22.5	72.8	3.5

[a]Compositions are given as percentages of each type of chain. Sum of unknown components was 24.2% for alkyl and 1.2% for fatty acid.

first time. Skin surface lipids have previously been analyzed in several mammalian species, including the mouse. Nicolaides *et al.* (11) had reported that there are two types of diol diester waxes in the skin surface lipids of the mouse, namely a slower and a faster migrating fraction in TLC, although no structural evidence was presented. The lesser mobility of the slower migrating fraction was attributed to the abundance of unsaturated fatty acids and branched-chain alkanediol.

The skin lipid profile of the hairless mouse shown in the present study is quite similar to that described by Nicolaides *et al.* (11) for the mouse. On the basis of its mobility in TLC, the lipid identified as alkyldiacylglycerol in the present study appeared to correspond to the lower mobility diester wax

TABLE 2
Diol and Fatty Acid Compositions of Diester Wax[a]

Carbon number	Diol	Fatty acid			
		Straight		Branched-	
	Straight	Saturated	Monoenoic	*iso*	*anteiso*
15		0.2		0.1	
16	11.4	5.6	6.3	1.2	
17		0.6		0.4	0.7
18	3.8	3.2	7.7	2.3	
19		2.9		0.9	0.3
20	47.6	13.4	10.8	6.2	
21		4.0		2.7	0.7
22	8.8	7.3	11.9	5.2	
23		0.7		0.6	0.1
24		1.4	1.2	0.5	
Sum	71.6	39.3	37.9	20.1	1.8

[a]Compositions are given as percentages of each type of chain. Sum of unknown component was 28.4% for diol and 0.8% for fatty acid.

which the earlier authors considered a subclass of diacyl alkanediol. It has been noted that a certain diversity exists in the skin surface lipids between different animal species (11,12). Reevaluation of these data suggests that the skin surface lipids of several animals seem to contain the lipid class with a mobility in TLC similar to that of diacylated glycerol ethers. Thus, glycerol ether diesters may be rather common on the skin surface of animals.

Although little is known about the physiological significance of the ether lipid, the presence of an ether linkage in its structure may hinder hydrolysis by the bacterial enzymes present on the skin surface. The relative stability of these compounds, would also explain the lower levels of free fatty acids in the skin surface lipids of animals.

REFERENCES

1. Nicolaides, N. (1971) *Lipids 6,* 901–905.
2. Nikkari, T. (1965) *Scand. J. Clin. Lab. Invest. (Suppl. 85) 17,* 1–140.
3. Wilkinson, D.I., and Karasek, M.A. (1966) *J. Invest. Dermatol. 47,* 449–455.
4. Sharaf, D.M., Clark, S.J., and Downing, D.T. (1977) *Lipids* 12, 786–790.
5. Downing, D.T., and Colton, S.W. (1980) *Lipids 15,* 323–327.
6. Downing, D.T., and Sharaf, D.M. (1976) *Biochim. Biophys. Acta 431,* 378–389.
7. Oku, H., Nakanishi, T., Kumamoto, K., and Chinen, I. (1990) *Comp. Biochem. Physiol. 96B,* 475–482.
8. Harvey, D.J. (1982) *Biomed. Mass. Spectrom. 9,* 33–38.
9. Harvey, D.J. (1992) in *Advances in Lipid Methodology I* (Christie, W.W., ed.) pp. 19–80, The Oily Press Ltd., Ayr.
10. Harvey, D.J. (1991) *Biol. Mass Spectrom.* 20, 87–93.
11. Nicolaides, N., Fu, H.C., and Ansari, M.N.A. (1970) *Lipids* 5, 299–307.
12. Lindholm, J.S., McCormick, J.M., Colton VI, S.W., and Downing, D.T. (1981) *Comp. Biochem. Physiol. 69B,* 75–78.

[Received June 22, 1993, and in final revised form December 17, 1994; Revision accepted January 12, 1995]

Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization Mass Spectrometry

Wm. Craig Byrdwell* and Edward A. Emken

FQS, NCAUR, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: Atmospheric pressure chemical ionization (APCI) mass spectrometry was investigated as a new method for analysis of a mixture of triglycerides separated by reverse-phase high-performance liquid chromatography. A mixture of homogeneous (monoacid) triglyceride standards containing fatty acids with zero to three double bonds was analyzed to demonstrate the quality of mass spectra obtained by using the APCI interface. The mass spectra showed that minimal fragmentation occurs, resulting primarily in diglyceride $[M - RCOO]^+$ ions and $[M + 1]^+$ protonated molecular ions. The degree of unsaturation within the acyl chains had a marked effect on the proportion of diglyceride ions vs. the $[M + 1]^+$ ions formed in the APCI source. The mass spectra of triglycerides containing fatty acids with two or three double bonds showed predominantly protonated triglyceride ions, with diglyceride peaks representing 13 to 25% of the base peak. The triglycerides containing singly unsaturated fatty acids gave diglyceride ions as the base peak, and $[M + 1]^+$ ions with an intensity 20 to 28% that of the base peak. Only diglyceride ions were observable in the spectra of triglycerides containing saturated fatty acids.

Lipids 30, 173–175 (1995).

Separated (1–6), fractionated (7–9) and unseparated (10–20) mixtures of triglycerides (TG) have been analyzed using direct inlet introduction (1–4,6), desorption chemical ionization (9,19, 20), direct insertion probe introduction (7,8,10–14), electrospray (18) and other (5,15–17) mass spectrometric methods. The use of electron impact ionization methods for analysis of TG generally results in spectra containing low-molecular weight fragments, with no quasimolecular ions present. Spectra of TGs obtained using desorption chemical ionization methods show increased amounts of quasi-molecular ions, while retaining moderate fragmentation, which can be useful for structural assignment. Desorption chemical ionization, however, is not amenable to use for direct detection in a high-performance liquid chromatography (HPLC) flow system. Direct insertion probe methods can provide spectra containing minimal fragmentation, with primarily diglyceride and TG ions. These spectra may readily allow identification of a number of molecular species of TG. However, direct probe introduction is also not amenable to direct detection in a flow system. Direct inlet introduction allows direct detection in an HPLC system and also produces spectra that contain sizeable quasimolecular ions and minimal fragmentation. It has been demonstrated to be an effective interface for TG analysis (1–4,6). Electrospray ionization (ESI) has been used for analysis of a mixture of TGs infused *via* syringe pump (18). Spectra obtained using ESI contained only quasimolecular ions, with no fragmentation. This lack of fragmentation can result in ambiguity in structural assignments for TG of identical molecular weight. Tandem mass spectrometry/mass spectrometry (MS/MS) could provide the additional structural information necessary to uniquely identify TG species. Collision-induced dissociation can also be used to produce lower molecular weight ions. The flow rates used for syringe pump infusion are very low compared to those in a conventional HPLC flow system. The necessity of an ionic buffer in the effluent for ESI will affect the types of columns and solvents which may be used. Alternatively, a sheath liquid may be used to provide the necessary ionic buffer. In either case, the ESI–MS detectability is based on the ability to ionize the neutral TG in solution. So, while preliminary results of use of ESI for detection of nonpolar TG are encouraging, further data will be necessary for a thorough comparison of ionization methods in an HPLC system.

Atmospheric pressure chemical ionization (APCI), like ESI, is an atmospheric pressure ionization method (21). However, unlike ESI, APCI uses a corona needle discharge to impart charge onto vaporized molecules which are sprayed from a capillary inlet. These are swept into the high vacuum region of the mass spectrometer through a capillary bleed. Also unlike ESI, APCI requires no buffers in solution in order to produce efficient fragmentation. This lack of need of a buffer allows neutral, nonpolar molecules to be analyzed as easily as more polar molecules which are ionizable in solution. An APCI interface allows direct introduction of HPLC column effluent at a rate of up to 2 mL/min.

Presented here is the first report of an APCI interface for the direct mass spectrometric analysis of TG standards. We demonstrate that, because of minimal fragmentation, APCI is

*To whom correspondence should be addressed at FQS, USDA, ARS, NCAUR, 1815 N. University St., Peoria, IL 61604.

Abbreviations: APCI, atmospheric pressure chemical ionization; DG, diglyceride; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MS, mass spectrometry; RIC, reconstructed ion chromatogram; RP-HPLC, reverse-phase high-performance liquid chromatography; TG, triglyceride.

Copyright © 1995 by AOCS Press

an effective method for identification of TG species separated by reverse phase HPLC (RP-HPLC).

MATERIALS AND METHODS

TG. Individual TG standards were obtained from Nu-Chek-Prep (Elysian, MN). A TG mixture was made by dissolving 25–37 mg of each TG (liquid or solid) in 5 mL of tetrahydrofuran, for a total concentration of 57.0 mg/mL. The following homogeneous (monoacid) TG were included in the mixture: tripalmitin (tri 16:0, PPP), tripalmitolein (tri 16:1, PoPoPo), tristearin (tri 18:0, SSS), triolein (tri 18:1, OOO), trilinolein (tri 18:2, LLL), trilinolenin (tri 18:3, LnLnLn), triarachidin (tri 20:0, AAA), trigadolein (tri 20:1, GGG) and trierucin (tri 22:1, EEE).

Chromatography. All solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents were HPLC grade or the highest available quality and were used without further purification. The HPLC pump was an LDC 4100 MS (Thermo Separation Products, Schaumburg, IL) quaternary pump system with membrane degasser. The columns used were an Adsorbosphere C18 (Alltech Associates, Deerfield, IL), 25 cm × 4.6 mm, 5 μm (12% carbon load) in series with an Adsorbosphere UHS C18 25 cm × 4.6 mm, 10 μm (30% carbon load). A gradient solvent program with propionitrile (PrCN) and hexane (Hex) was used to accomplish the separations. The gradient used was as follows: initial 88% PrCN, 12% Hex; linear from 10 to 15 min to 75% PrCN, 25% Hex, held until 25 min; linear from 25 to 30 min to 50% PrCN, 50% Hex, held until 40 min. The flow rate was 1 mL/min. The flow was split so that ~600 μL/min went to an evaporative light-scattering detector (ELSD) and ~400 μL/min went to the APCI interface. 5 μL of the TG mixture was injected, which resulted in ~114 μg of total TG mixture (10 to 15 μg of each component) going to the APCI interface. High signal-to-noise ratios in the mass spectra indicate that usable spectra may be obtainable using perhaps a hundred-fold less sample. However, the total ion chromatographic signal-to-noise ratio is much lower, so smaller samples necessitate greater reliance upon the ELSD chromatogram.

The ELSD was an ELSD MKIII (Varex, Burtonsville, MD). The drift tube was set at 140°C, the gas flow was 2.0 standard liters per minute. High-purity N_2 was used as the nebulizer gas.

MS. A Finnigan MAT (San Jose, CA) SSQ 710C fitted with an APCI source was used to acquire mass spectral data. The vaporizer was operated at 240°C; the capillary heater was operated at 250°C. The corona voltage was set at 5 μA. High-purity nitrogen was used for the sheath and auxiliary gases, which were set to 55 psi and 5 mL/min, respectively. Spectra were obtained from 400 to 1100 amu, with a scan time of 2.5 s. Preliminary data showed no substantial ions below 400 amu. The total ion chromatogram was processed using the data system's automatic seven-point smoothing and peak label functions. A peak threshold of 1% intensity was applied to the mass spectra.

RESULTS AND DISCUSSION

The reconstructed ion chromatogram (RIC) of the separated TG mixture is presented in Figure 1. The chromatogram obtained from the ELSD is similar to the RIC and is not presented here. Representative mass spectra of monoacid TG containing 3, 2, 1 and 0 sites of unsaturation in the acyl chains are shown in Figure 2. The mass spectra of the TG showed very little fragmentation. The most significant fragments present in these spectra arose from the protonated molecular ion $[TG + 1]^+$ and the diglyceride (DG) ion $[M - RCOO]^+$, or $[DG]^+$. The TG that contained two or more sites of unsaturation (LnLnLn and LLL) in the fatty acyl chains gave a $[TG + 1]^+$ base peak. The $[DG]^+$ peak of those TG had an intensity of approximately 20 to 35% that of the base peak. Those TG containing less than two sites of unsaturation in each acyl chain showed a base peak arising from the DG ion. TG that contained one site of unsaturation in each of the acyl chains (PoPoPo, OOO, GGG, EEE) showed a $[DG]^+$ base peak, with a $[TG + 1]^+$ peak intensity which was 15 to 20% that of the base peak. Completely saturated TG produced no detectable $[TG + 1]^+$ peak. The spectra of these TG consisted solely of $[DG]^+$ fragments. Spectra of most TG showed minor peaks representing the addition of water to the DG and/or TG ion, $[DG + 18]^+$ and/or $[TG + 1 + 18]^+$. Adduct formation of some TG with the propionitrile solvent was also observed with the appearance of $[TG + 1 + 55]^+$ ions.

The variation with respect to molecular ion and fragment ion abundances that we observed in these spectra due to varying degrees of unsaturation has been observed using other ionization methods (1,2,8,11,19). This variation in ion intensities has necessitated the use of response factors, or calibration factors, for quantitation. Quantitation from spectra obtained with APCI will likewise require the use of calibration factors. For qualitative analysis, however, APCI spectra allowed direct identification of numerous TG species.

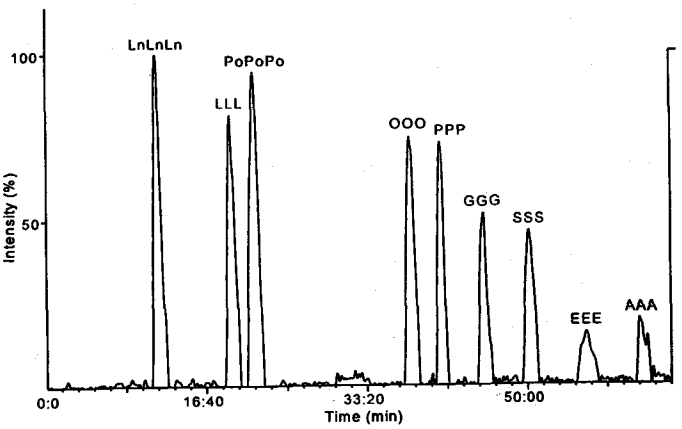


FIG. 1. Total ion chromatogram of triglyceride mixture. Order of elution: trilinolenin (LnLnLn), trilinolein (LLL), tripalmitolein (PoPoPo), triolein (OOO), tripalmitin (PPP), trigadolein (GGG), tristearin (SSS), trierucin (EEE), triarachidin (AAA).

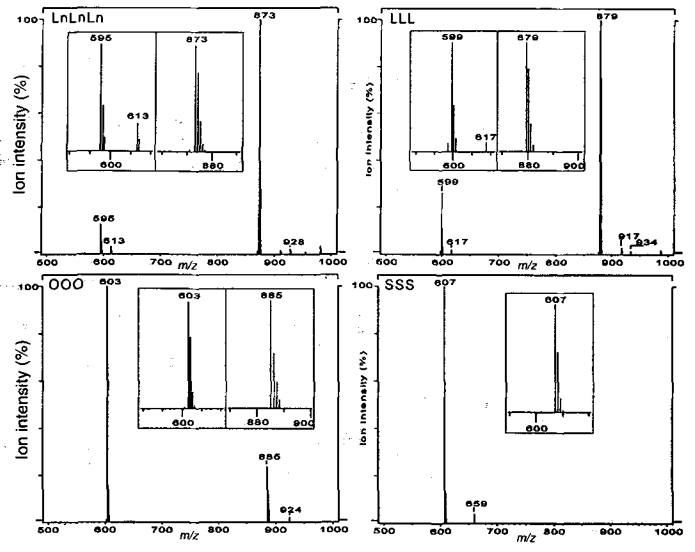


FIG. 2. Mass spectra of LnLnLn, LLL, OOO and SSS. See Figure 1 for abbreviations.

These results clearly show the utility of APCI as an ionization method for TG analysis. The compatibility of APCI with a nonpolar chromatographic solvent system allows an expanded range of molecules to be analyzed, compared to the other widely used atmospheric pressure ionization method, ESI. While the APCI source did not give molecular ions as does ESI for trisaturated TG, the simple fragmentation pattern of all species allowed identification by the DG fragments.

REFERENCES

1. Kuksis, A., Marai, L., and Myher, J.J. (1983) *J. Chromatogr. 273*, 43–66.
2. Myher, J.J., Kuksis, A., Marai, L., and Manganaro, F. (1984) *J. Chromatogr. 283*, 289–301.
3. Kuksis, A., Myher, J.J., and Marai, L. (1985) *J. Am. Oil Chem. Soc. 62*, 762–767.
4. Kuksis, A., and Myher, J.J. (1986) *J. Chromatogr. 379*, 57–90.
5. Kallio, H., Laakso, P., Huopalahti, R., Linko, R.R., and Olsman, P. (1989) *Anal. Chem. 61*, 698–700.
6. Kuksis, A., Marai, L., and Myher, J.J. (1991) *Lipids 26*, 240–246.
7. Merritt Jr., C., Vajdi, M., Kayser, S.G., Halliday, J.W., and Bazinet, M.L. (1982) *J. Am. Oil Chem. Soc. 59*, 422–432.
8. Rezanka, T., Husek, P., and Podojil, M. (1986) *J. Chromatogr. 355*, 265–271.
9. Singleton, J.A., and Pattee, H.E. (1987) *J. Am. Oil Chem. Soc. 64*, 534–538.
10. Barber, M., Merren, T.O., and Kelly, W. (1964) *Tetrahedron Lett.*, 1063–1067.
11. Hites, R.A. (1970) *Anal. Chem. 42*, 1736–1740.
12. Aasen, A.J., Lauer, W.M., and Holman, R.T. (1973) *Lipids 5*, 869–877.
13. Lauer, W.M., Aasen, A.J., Graff, G., and Holman, R.T. (1973) *Lipids 5*, 861–868.
14. Murata, T., and Takahashi, S. (1977) *Anal. Chem. 49*, 728–731.
15. Lehmann, W.D., and Kessler, M. (1983) *Biomed. Mass Spectrom. 10*, 220–226.
16. Barber, M., Tetler, L.W., Bell, D., Ashcroft, A.E., Brown, R.S., and Moore, C. (1987) *Org. Mass Spectrom. 22*, 647–650.
17. Showell, J.S., Fales, H.M., and Sokoloski, E.A. (1989) *Org. Mass Spectrom. 24*, 632–636.
18. Duffin, K.L., Henion, J.D., and Shieh, J.J. (1991) *Anal. Chem. 63*, 1781–1788.
19. Mares, P., Rezanka, T., and Novak, M. (1991) *J. Chromatogr. 568*, 1–10.
20. Rezanka, T., and Mares, P. (1991) *J. Chromatogr. 542*, 145–159.
21. Covey, T.R., Lee, E.D., Bruins, A.P., and Henion, J.D. (1986) *Anal. Chem. 58*, 1451A–1461A.

[Received on September 27, 1994, and in revised form December 16, 1994; Revision accepted on December 28, 1994]

Incorporation of *all-trans*- or 9-*cis*-β-Carotene into Mixed Micelles *in vitro*

Galit Levin and Shoshana Mokady*

Department of Food Engineering and Biotechnology, Technion—Israel Institute of Technology, Haifa 32000, Israel

ABSTRACT: Various studies suggest that *all-trans*- and 9-*cis*-β-carotene are absorbed in the intestine to different extents. The present study was undertaken to evaluate the degree of *in vitro* incorporation of the two isomers into intestinal mixed micelles, which is an essential early step in the absorption process. The micelles were designed to simulate those present during fat digestion in the lumen of the human small intestine with respect to bile salts, lipids, pH and temperature. Solutions of *all-trans*- and 9-*cis*-β-carotene at various ratios were added to the lipid mixture. A direct correlation was seen between the 9-*cis*-β-carotene level in the mixture and the degree of total β-carotene incorporation into micelles. An increased level of *all-trans*-β-carotene in the system led to a decrease in the percentage of β-carotene incorporated into the micelles. In contrast, when carotene mixtures enriched with the 9-*cis* isomer were used, an increase in the level of total carotene in the solution was accompanied by a constant or even enhanced carotene incorporation. The results indicate that the differences in the absorption of β-carotene isomers might be the result of their different ability to be incorporated into the lipid micelles of the intestinal lumen. In addition, the results point toward the possibility that ingestion of 9-*cis*-β-carotene by humans may increase carotene availability.

Lipids 30, 177–179 (1995).

In recent years β-carotene has been suggested to be an important antioxidant and anticancer agent (1). Due to its hydrophobic nature, β-carotene is incorporated in the intestine into the lipid mixed micelles, and then absorbed (2). The efficiency of *all-trans*-β-carotene absorption in humans is quite low (10–15%) (3).

Fruits and vegetables are known to contain, in addition to *all-trans*-β-carotene, varying amounts of the 9-*cis* isomer (4). In our laboratory, the metabolism of the 9-*cis*-β-carotene stereoisomer in living organisms is being studied using the alga *Dunaliella bardawil* as a source of 9-*cis*-β-carotene (5–7). We have recently shown that the 9-*cis* isomer may possess better antioxidant properties than the *all-trans* isomer (8). Therefore, its absorption characteristics in the small intestine, especially in humans, is of considerable interest.

*To whom correspondence should be addressed.

Abbreviations: HPLC, high-performance liquid chromatography; THF, tetrahydrofuran.

Previous studies had indicated that there are species-specific differences in the ability to absorb various carotenoids (9). It is now well accepted that the solubilization of carotenoids in intestinal lipid micelles is a prerequisite for their absorption. The species differences in carotenoid absorption might therefore be due to the ability in a given species to form a suitable micellar solution in the intestinal lumen with one carotenoid but not with the other (9). We recently suggested (7) that there are differences between rats and chicks in the absorption pattern of *all-trans*- and 9-*cis*-β-carotene. However, no information is available in regard to these luminar events in humans.

In the present study, the incorporation of *all-trans*- and 9-*cis*-β-carotene into mixed micelles was examined *in vitro.* Conditions were maintained similar to those thought to be present in the lumen of the human small intestine during fat digestion and absorption with respect to bile salts, Na^+, lipid concentration, pH and temperature (10).

MATERIALS AND METHODS

Carotene extraction and purification. Spray-dried *D. bardawil* (NBT, Eilat, Israel) was extracted with a mixture of 30% ethanol (95%) and 70% hexane (vol/vol), followed by three additional extractions with hexane. Following evaporation of the solvents and saponification, the pigments were transferred into hexane, washed with water, and the extract was filtered to remove crystals of *all-trans*-β-carotene. The extract was further purified (8) by high-performance liquid chromatography (HPLC) on a C18 reverse-phase preparative column and a guard column (218TP1022 and 201FSK54 respectively; Vydac, The Separation Group, Hesparia, CA). The product obtained was composed of 85.7% 9-*cis*- and 14.3% *all-trans*-β-carotene. The carotenes were taken up in tetrahydrofuran (THF) and brought to 1.6 mM. Synthetic *all-trans*-β-carotene (Type I; Sigma, St. Louis, MO) was dissolved in THF at the same concentration. Mixtures of the two β-carotene solutions with 9-*cis*-β-carotene concentrations of 5, 23, 47.5, 62.5 and 85.7% (wt/vol) were prepared.

Preparation of mixed micelles. Mixed micelles containing the β-carotenes were prepared by the method of Canfield *et al.* (11). Briefly, a solution of triolein, monoolein, oleic acid and dipalmitoyl phosphatidylcholine in hexane was mixed

Copyright © 1995 by AOCS Press

with one of the above carotene solutions. The levels of total β-carotene in the solutions were 40, 80, 120, 160, 200 and 240 μM. Following evaporation of the solvent, a mixture of bile acids in Krebs-Ringer bicarbonate buffer (pH 7.4) was added. Sonication was carried out at 37°C for 2 h using a bath sonicator (E/MC Fisher, Scientific International, Springfield, NJ). The solution was then centrifuged (106000 × *g*) for 2 h using a Centricon T-2055 ultracentrifuge (Kontron Analytical, Zurich, Switzerland). Solubilization of β-carotene in the lower aqueous phase was taken as evidence for micelle formation (11).

Carotene analysis. Carotene was extracted from the micelles with hexane/ethanol (3:1, vol/vol) (11). The concentration of total β-carotene was determined spectrophotometrically at 472 nm ($E^{1\%}_{1\ cm} = 2262$). The isomeric β-carotene composition in the micelles was assayed by HPLC using an analytical column as described before (7).

Statistical analysis. Differences between group means were determined by Student's *t*-test.

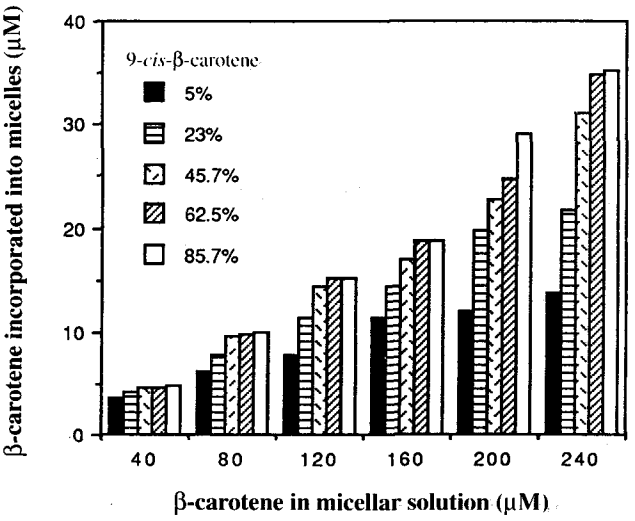


FIG. 1. Effect of 9-*cis*-β-carotene concentration in systems containing various amounts of total β-carotene on the level of total β-carotene incorporated into mixed micelles *in vitro*.

RESULTS

The level of incorporation of β-carotenes into lipid mixed micelles was studied in an *in vitro* system. Larger amounts of β-carotene were incorporated into the micelles when the carotene mixtures contained 85.7% rather than 5% 9-*cis*-β-carotene (Table 1). The differences observed were highly significant at all β-carotene concentrations tested. This enhancement, due to the higher level of 9-*cis*-β-carotene in the solution, was also seen in systems containing identical amounts of total carotene (Fig. 1).

When *all-trans*-β-carotene (5% 9-*cis*-β-carotene) was used at higher levels, the amount of β-carotene that was incorporated into the micelles reached a plateau at approximately 14 μM. However, when carotene mixtures with increasing concentrations of the 9-*cis* isomer were used, a maximal carotene level in the micelles was not reached. On the contrary, an increase in micellar carotene content was observed. This enhancement was most pronounced at the two highest total carotene levels (Fig. 1).

Increased levels of *all-trans*-β-carotene in the solution led to a decrease in the percentage of β-carotene incorporated into the micelles (Fig. 2). In contrast, when the various carotene mixtures enriched with the 9-*cis* isomer were used, an increase in the level of total carotene in the solution was accompanied by a constant or even an enhanced efficiency of β-carotene incorporation.

The isomeric compositions of the micellar β-carotenes was found to be identical to those of the carotene mixtures in the solutions (Fig. 3).

TABLE 1
Incorporation of Total β-Carotene into Mixed Micelles as a Function of Isomeric Composition

β-Carotene concentration in micellar solution (μM)	β-Carotene in micelles (μM)[a]	
	5% 9-*cis*-β-carotene	85.7% 9-*cis*-β-carotene
80	5.85 ± 0.63	9.42 ± 0.37[b]
200	13.73 ± 1.56	26.39 ± 1.58[b]
240	16.28 ± 1.77	37.36 ± 2.64[b]

[a]Mean ± SD of three samples.
[b]Statistically significant effect ($P < 0.01$) of 9-*cis*-β-carotene concentration.

DISCUSSION

In the present study we found that the isomeric composition of the β-carotene mixtures affected the amount of total carotene incorporated into the lipid mixed micelles, simulating those formed in human small intestine. Specifically, the level of incorporation was found to increase in relation with the amount of 9-*cis*-β-carotene in the mixture.

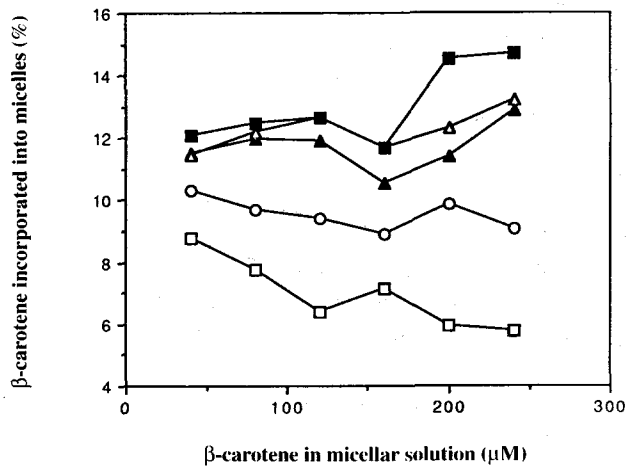


FIG. 2. Efficiency (%) of incorporation of β-carotene into mixed micellar solutions in systems containing various amounts of total β-carotene as a function of 9-*cis*-β-carotene concentration: 5% (□); 23% (○); 45.7% (▲); 62.5% (△); or 85.7% (■).

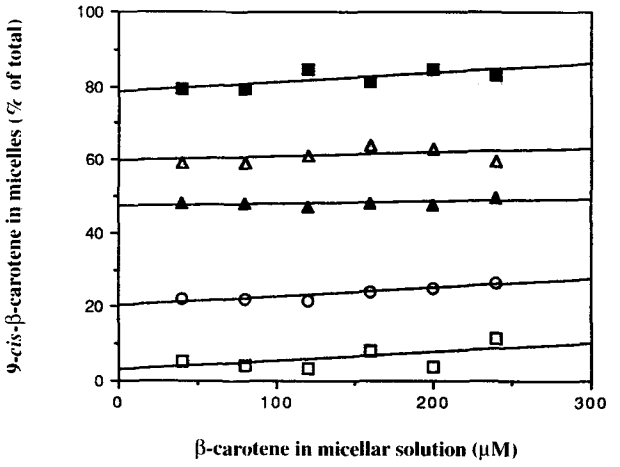


FIG. 3. Isomeric composition of micellar β-carotene in systems containing various amounts of total β-carotene as a function of 9-*cis*-β-carotene concentration: 5% (□); 23% (○); 45.7% (▲); 62.5% (△); or 85.7% (■).

The incorporation of β-carotene into mixed micelles has previously been studied only with *all-trans*-β-carotene. Thus, Canfield *et al.* (11) have shown that the incorporation efficiency decreased with higher amounts of β-carotene in the solution, reaching an upper level of 15 μM, which is in good agreement with the value of 14 μM obtained in the present study.

In experiments with rats and chicks, it was shown that a higher concentration of 9-*cis*-β-carotene in the diet was associated with a reduction in the hepatic carotene levels in rats, while in chicks an increase was observed (7). This was explained by possible differences between the two animal species in the micellar composition, and consequently in the ability to incorporate the various β-carotene isomers, which resulted in differences in the extent of absorption of the isomers. Since a species difference apparently exists, it was not possible to predict the efficiency of the absorption of the two isomers in humans. The present *in vitro* study suggests that 9-*cis*-β-carotene in the human diet might bring about an increase in the amount of total carotene incorporated into micelles, and thus cause an increase in carotene absorption similar to what has been observed in chicks. However, in contrast to chicks in which the hepatic and possibly the micellar 9-*cis* isomer concentration was higher than in the diet, in the micelles simulating those formed in the human intestine, the level of the 9-*cis* isomer was similar to that in the solution.

Although the present study suggests that 9-*cis*-β-carotene can be incorporated into intestinal micelles quite efficiently, it is of importance to note that the absorption of 9-*cis*-β-carotene in the human small intestine has previously been questioned. Jensen *et al.* (12) studied the levels of *all-trans*- and 9-*cis*-β-carotene in human serum following ingestion of *Dunaliella* carotene extract, or carrot extract which contained mostly the *all-trans* isomer. In both dietary treatments, the major isomer found in the serum was *all-trans*-β-carotene, and the ratio between the two isomers was identical. These researchers thus concluded that in humans there is a preferential absorption of the *all-trans*-β-carotene over that of the 9-*cis* isomer. Stahl *et al.* (13) in a more recent study was not able to show the presence of 9-*cis*-β-carotene in human serum following *Dunaliella* consumption. However, analyses of β-carotene isomeric composition in human livers revealed the presence of substantial amounts of 9-*cis*-β-carotene (14). This observation might point to the possibility that 9-*cis*-β-carotene is absorbed in humans but is immediately transferred to and accumulated in tissues and thus is not found in the serum (14). The results of the present study, in which mixed micelles were shown to contain substantial amounts of 9-*cis*-β-carotene, are consistent with this hypothesis. Nonetheless, further experiments are needed to clarify these points.

ACKNOWLEDGMENT

The technical support of the Otto-Meyerhof Biotechnological Laboratories, established by the Minerva Foundation, is acknowledged.

REFERENCES

1. Gerster, H. (1993) *Internatl. J. Vit. Nutr. Res. 63,* 93–121.
2. Tee, E.S. (1992) *Critical Rev. Food Sci. and Nutr. 31,* 103–163.
3. Singh, V.N. (1992) in *Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications* (Ong, A.S.H., and Packer, L., eds.) pp. 208–227, Birkhäuser Verlag, Basel.
4. Chandler, L.A., and Schwartz, S.J. (1987) *J. Food Sci. 52,* 669–672.
5. Mokady, S. (1992) in *Dunaliella: Physiology, Biochemistry and Biotechnology* (Avron, M., and Ben-Amotz, A., eds.) pp. 217–229, CRC Press, Boca Raton.
6. Levin, G., and Mokady, S. (1994) *Internatl. J. Vit. Nutr. Res. 64,* 165–169.
7. Levin, G., Ben-Amotz, A., and Mokady, S. (1994) *Comp. Biochem. Physiol. 107A,* 203–207.
8. Levin, G., and Mokady, S. (1994) *Free Radical Biol. Med. 17,* 77–82.
9. Bauernfeind, J.C., Adams, C.R., and Marusich, W.L. (1981) in *Carotenoids as Colorants and Vitamin A Precursors* (Bauernfeind, J.C., ed.) pp. 563–743, Academic Press, New York.
10. El-Gorab, M., and Underwood, B.A. (1973) *Biochim. Biophys. Acta 306,* 58–66.
11. Canfield, L.M., Fritz, T.A., and Tarara, T.E. (1990) *Methods Enzymol. 189,* 418–422.
12. Jensen, C.D., Howes, T.M., Spiller, G.A., Pattison, T.S., Whittam, J.H., and Scala, J. (1987) *Nutr. Rep. Int. 35,* 413–423.
13. Stahl, W., Schwarz, W., and Sies, H. (1993) *J. Nutr. 123,* 847–851.
14. Stahl, W., Sundquist, A.R., Hanusch, M., Schwartz, W., and Sies, H. (1993) *Clin. Chem. 39,* 810–814.

[Received June 1, 1994, and in revised form December 19, 1994; Revision accepted December 27, 1994]

Effect of Growth Temperature on the Positional Distribution of Eicosapentaenoic Acid and *trans* Hexadecenoic Acid in the Phospholipids of a *Vibrio* Species of Bacterium

R.J. Henderson*, R.-M. Millar and J.R. Sargent

N.E.R.C. Unit of Aquatic Biochemistry, Department of Biological and Molecular Sciences, University of Stirling, Stirling FK9 4LA, Scotland

ABSTRACT: Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were isolated from a *Vibrio* species of bacterium, known to produce eicosapentaenoic acid (20:5n-3) and *trans*-hexadecenoic acid (16:1n-7), and subjected to phospholipase A_2 degradation to determine the positional distribution of component fatty acids. At the two growth temperatures studied (20 and 5°C), both 20:5n-3 and *trans* 16:1 n-7 were located mainly at position *sn*-2 in PE. Increases in the proportions of 20:5n-3 and *trans* 16:1n-7 in position *sn*-2 with decreasing growth temperature were balanced mainly by decreases in the level of *iso*-15:0. In PG, *trans* 16:1n-7 was located predominantly in position *sn*-1, although the difference between the two positions was not as great as in PE. Eicosapentaenoic acid was preferentially located in position *sn*-2 of PG, particularly at 5°C when it comprised 29.9% of the total fatty acids in this position. It is concluded that *trans* 16:1n-7/20:5n-3 is not a major molecular species of phospholipid in this species of *Vibrio* and that changes in the levels of molecular species of PE containing *iso*-15:0 may feature in thermal acclimation.

Lipids 30, 181–185 (1995).

It is well established that alterations in membrane lipid composition occur in poikilothermic organisms as an adaptation to changes in the temperature of their environment (1,2). An increase in the level of polyunsaturated fatty acids (PUFA) in phospholipids is an almost ubiquitous response in poikilothermic eukaryotes to a decrease in temperature. Bacteria do not, in general, contain PUFA (3). Temperature-induced adaptations in the fatty acid profile of bacteria that have been noted include increases in the proportions of monounsaturated fatty acids, shorter chain fatty acids and branched-chain fatty acids, as well as changes in the ratio of *anteiso*- to *iso* acids (4–6). Some strains of bacteria contain *trans*-monounsaturated fatty acids in addition to the more common *cis* isomers, and it has been shown that in the psychrophilic bacterium *Vibrio* sp. strain ABE-1, the relative proportion of *trans* 16:1n-7 decreases as a response to a decrease in environmental temperature (7,8).

We have recently reported the simultaneous occurrence of eicosapentaenoic acid (20:5n-3) and *trans* hexadecenoic acid (16:1n-7) in a *Vibrio* species of bacterium isolated from fish intestines and have shown that the level of both these fatty acids in the cellular phospholipids is higher at a growth temperature of 5 than 20°C (9). This increase in the level of *trans* 16:1 with decreasing growth temperature contrasts with the decrease observed with *Vibrio* ABE-1 species (7,8). In the latter species the *trans* fatty acid is known to be located mainly in position *sn*-2 of phosphatidylethanolamine (PE) (8). Although position *sn-2* of the phospholipids of most eukaryotes is characteristically enriched in PUFA (10,11), the C_{18} PUFA of photosynthetic cyanobacteria are known to be specifically located in the *sn*-1 position of phosphatidylglycerol (PG) (12). No information is available for the positional distribution of 20:5n-3 in the phospholipids of bacteria. We have previously postulated that the observed increase in both *trans* 16:1n-7 and 20:5n-3 in the *Vibrio* species with decreasing temperature results from the specific increase in a molecular species of phospholipid containing both these two fatty acids (9), a suggestion which implies that the fatty acids occupy different positions within the phospholipid. The study described here was undertaken to determine the positional distribution of 20:5n-3 and *trans* 16:1n-7 in the phospholipids of the *Vibrio* species of bacterium, which contains both these fatty acids. In addition to determining how the two fatty acids are distributed within the two constituent phospholipids of the *Vibrio* species, PE and PG (9), it was intended to establish whether specific changes in positional distribution occur in response to changes in growth temperature.

MATERIALS AND METHODS

Chemicals. Inorganic chemicals were of AnalaR grade and were supplied by BDH (Lutterworth, Leics., United Kingdom). Solvents were of glass-distilled or high-performance

*To whom correspondence should be addressed.

Abbreviations. HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; UV, ultraviolet.

Copyright © 1995 by AOCS Press

liquid chromatography (HPLC) grade and were obtained from Rathburn Chemicals (Walkerburn, United Kingdom). Phospholipase A_2 (E.C. 3.1.1.4) prepared from *Naja naja* venom was purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom).

Growth of bacteria. The bacterium studied was the *Vibrio* species originally isolated from the gut of Arctic char *(Salvelinus alpinus* L.) (13). Cultures of the bacterium were grown under sterile conditions in defatted, freshwater medium in the dark at 20 and 5°C as described previously (9), and harvested during late log phase (24 h and 5 d for cultures at 20 and 5°C, respectively) by centrifugation at 7,000 × *g* for 10 min. Cultures were grown sequentially and did not arise from the same inoculating culture.

Phospholipid analysis. Lipid was extracted from pellets of bacterial cells by blending with chloroform/propan-2-ol (2:1, vol/vol) as described previously (9). All lipid extracts and fractions were maintained at −70°C under an atmosphere of oxygen-free nitrogen between analytical operations. PE and PG were isolated by thin-layer chromatography (TLC) of total lipid extracts on 20 × 20 cm glass plates precoated with silica gel G (E. Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, by vol) as developing solvent. Developed chromatograms were sprayed lightly with 0.01% (wt/vol) 2′,7′-dichlorofuorescein in methanol and viewed under ultraviolet (UV) light. Bands of adsorbent containing PE and PG were scraped separately from the plates and the phospholipids eluted from the adsorbent with chloroform/methanol/water (5:5:1, by vol). After removal of the solvent by evaporation and desiccation under vacuum, the phospholipids were redissolved in chloroform/methanol (2:1, vol/vol) and the purity of aliquots checked by high-performance TLC (HPTLC) on silica gel using chloroform/methanol/water (65:25:4, by vol) as developing solvent.

For the enzymatic hydrolysis of phospholipids, 2.5 mg PE or PG were dissolved in 0.75 mL diethyl ether, and 0.25 mL 0.1M Tris HCl, pH 7.5 containing 4 mM $CaCI_2$ and 20 units of phospholipase A_2, was added. The mixture was stirred vigorously at 30°C for 4 h. Lipids were then extracted from the assay with chloroform/methanol (2:1, vol/vol). After reduction of solvent volume by evaporation, lipid extracts were applied to HPTLC plates which were subjected to single-dimension, double development with methyl acetate/propan-2-ol/chloroform/methanol/0.25% KCl (25:25:25:10:9, by vol) followed by hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) (14). Developed chromatograms were sprayed lightly with 0.01% (wt/vol) 2′,7′-dichlorofluorescein in methanol and viewed under UV light. Bands corresponding to lysoPE or lysoPG and free fatty acids were marked, scraped from the HPTLC plates into test tubes and subjected to acid-catalyzed transesterification with 1% sulfuric acid in methanol to produce the methyl ester derivatives of the constituent fatty acids (15). Aliquots of PE and PG were also subjected directly to acid-catalyzed transesterification. Fatty acid methyl esters were purified by HPTLC on silica gel plates using hexane/diethyl ether/glacial acetic acid (85:1 :1, by vol) as developing solvent and recovered from the adsorbent by elution with hexane/diethyl ether (1:1, vol/vol).

Analyses of fatty acid methyl esters were carried out by gas chromatography using a fused silica capillary column (25 m × 0.32 mm i.d. coated with BPX 70; S.G.E., Milton Keynes, United Kingdom). Sample application was by on-column injection, H_2 was used as carrier gas and the oven temperature was programmed to increase from 50 to 180°C during the course of an analysis. Separated methyl esters were identified by reference to authentic standards and by comparison with previously characterized samples of total fatty acids from the same species (9). The molar amounts of fatty acids were calculated by multiplying the area of each chromatographic peak by the appropriate arithmetic factor (15). The fatty acid compositions of the free fatty acid fraction and the lysophospholipid were considered to represent that of positions *sn-2* and *sn*-1, respectively, of the original intact phospholipid. The accuracy of the positional analysis was considered acceptable when the sum of the concentrations of a given fatty acid in positions *sn*-1 and *sn*-2, divided by two, was within 2% of the value for the fatty acid in the original phospholipid.

Statistical evaluation. Student's *t*-test was used for the statistical analysis of data, and the level of significance was defined as $P < 0.05$.

RESULTS

PE. The fatty acid composition of PE, the major phospholipid of the *Vibrio* (9), was significantly influenced by the temperature at which the bacteria were grown (Table 1). PE of bacteria grown at 5°C contained significantly higher levels of 20:5n-3 and *trans* 16:1n-7 and, conversely, lower levels of *iso*-14:0, *iso*-15:0, 17:0, 18:1n-9 and 18:1n-7 than that isolated from bacteria grown at 20°C. At both growth temperatures, more than half of the fatty acids esterified in position *sn*-1 of PE were saturated, whereas position *sn*-2 contained mainly monounsaturated fatty acids. The bulk of 20:5n-3 was always located in position *sn*-2, which also contained most of the *trans* 16:1n-7 present in PE.

Growth temperature influenced the fatty acid composition of both positions of PE. Bacteria grown at 5°C contained approximately threefold higher levels of both 20:5n-3 and *trans* 16:1n-7 in position *sn*-1 of PE than bacteria grown at 20°C, although these fatty acids together still accounted for only 2.7% of the fatty acids in this position at 5°C. In position *sn*-2, 20:5n-3 and *trans* 16:1n-7 comprised 9.6 and 17.7% of the fatty acids in this position at 5°C as compared to the lower corresponding values of 2.5 and 6.4% at 20°C. Conversely, the level of saturated fatty acids, particularly *iso*-15:0, in position *sn*-2 was lower at 5 than 20°C. The level of the major fatty acid in position *sn*-2, *cis* 16:1 n-7, was not significantly changed by growth temperature although its level in position *sn*-1 was higher at 5 than 20°C (13.8% vs. 7.7%).

PG. As with PE, the overall fatty acid composition of PG was influenced by growth temperature (Table 2). In particu-

TABLE 1
Effect of Growth Temperature on the Positional Distribution of Fatty Acids in Phosphatidylethanolamine of *Vibrio* sp.[a]

	5°C			20°C		
Fatty acids	Overall composition	Position *sn*-1	Position *sn*-2	Overall composition	Position *sn*-1	Position *sn*-2
i-13:0	0.7 ± 0.5	0.5 ± 0.5	5.0 ± 4.3	1.7 ± 1.0	0.3 ± 0.1	4.9 ± 07
13:0	0.7 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.8 ± 0.2	0.1 ± 0.0	0.3 ± 0.1
i-14:0	0.1 ± 0.1[f]	0.1 ± 0.1	0.3 ± 0.2[f]	0.4 ± 0.1	0.1 ± 0.1	0.8 ± 0.1
14:0	2.1 ± 0.7	3.2 ± 2.3	0.8 ± 0.1[f]	1.5 ± 0.3	2.1 ± 0.1	1.3 ± 0.2
14:1n-5	—	—	0.1 ± 0.1	—	—	0.1 ± 0.1
i-15:0	11.1 ± 1.5[f]	18.3 ± 4.4	7.6 ± 1.5[f]	17.1 ± 2.3	21.9 ± 1.0	18.6 ± 0.9
a-15:0	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.3
15:0	4.7 ± 0.8	8.8 ± 2.4	0.7 ± 0.2	4 3 ± 0 3	8.0 ± 0.5	0.8 ± 0.1
15:1n-6	2.0 ± 0.5[d]	1.3 ± 0.3[f]	2.2 ± 0.6[c]	1.3 ± 0.1	0.4 ± 0.1	1.9 ± 0.5
16:0	14.2 ± 1.0	26.4 ± 2.5	2.8 ± 1.1	15.6 ± 1.3	28.7 ± 1.8	2.1 ± 1.1
16:1n-9	1.6 ± 0.3	2.0 ± 0.3	1.7 ± 0.4[f]	1.6 ± 0.6	2.0 ± 0.1	2.5 ± 0.2
cis 16:1n-7	26.3 ± 2.4	13.8 ± 2.3[f]	39.7 ± 3.6	25.6 ± 1.6	7.7 ± 0.4	40.7 ± 1.0
trans 16:1n-7	9.9 ± 1.8[f]	2.1 ± 0.4[f]	17.7 ± 3.1[f]	2.2 ± 1.5	0.6 ± 0.1	6.4 ± 1.4
17:0	1.1 ± 0.3[f]	1.8 ± 0.4[f]	0.7 ± 0.7	2.4 ± 0.2	4.1 ± 0.4	—
17:1n-8	8.6 ± 1.4	7.8 ± 1.7	6.9 ± 1.3[f]	9.8 ± 1.1	6.5 ± 0.5	10.6 ± 0.9
17:1n-6	1.2 ± 0.2	1.7 ± 0.5	0.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.2	0.2 ± 0.0
18:0	2.1 ± 3.3	0.7 ± 0.3[f]	0.9 ± 0.3	1.2 ± 0.2	1.4 ± 0.2	0.7 ± 0.3
18: In-9	1.8 ± 0.4[f]	1.7 ± 0.6[d]	1.7 ± 0 4[b]	4.0 ± 0.5	2.9 ± 0.4	4.2 ± 2.6
18:1n-7	4.6 ± 0 7[b]	7 7 ± 2.4	0.6 ± 0.2[e]	5 3 ± 0 5	7.9 ± 0.6	1.0 ± 0.1
20:5n-3	7.0 ± 1.0[f]	0.6 ± 0.1[f]	9.6 ± 2.3[f]	1.8 ± 0.2	0.2 ± 0.1	2.5 ± 0.2
Total sat.	37.0 ± 2.6[f]	60.3 ± 7.5	19.5 ± 6.0[f]	45.4 ± 2.6	67.1 ± 1.6	30.0 ± 1.4
Total mono.	56.0 ± 1.7[f]	38.1 ± 6.9[f]	70.8 ± 3.8	50.8 ± 2.3	29.4 ± 1.7	67.5 ± 1.6
Total PUFA	7.0 ± 1.0[f]	0.6 ± 0.1[f]	9.6 ± 2.3[f]	1.8 ± 0.2	0.2 ± 0.1	2.5 ± 0.3
Unidentified	—	1.0 ± 0.2	0.1 ± 0.1	2.0 ± 0.1	3.3 ± 0.2	—

[a]Values are mol% and are means ± SD of six cultures. Values at 5°C with superscripts are significantly different from the corresponding 20°C value; [b]$P < 0.05$; [c]$P < 0.02$; [d]$P < 0.01$; [e]$P < 0.002$; [f]$P < 0.001$. *i-, iso; a-, anteiso;* sat., saturated fatty acids; mono.. monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

lar, the level of monounsaturated and polyunsaturated fatty acids were lower and higher, respectively, at 5 than 20°C. The level of saturated fatty acids, around 28% of total PG fatty acids, was not significantly affected by growth temperature.

Regardless of growth temperature, position *sn*-1 of PG was characterized by a high content of saturated fatty acids, particularly 16:0, and the monounsaturated fatty acids *cis* 16:1n-7 and 18:1n-7. Position *sn*-2 contained less saturated fatty acids than position *sn*-1, but had a higher content of PUFA, in the form of 20:5n-3, and was richer in *cis* 16:1n-7. More *trans* 16:1n-7 was located in position *sn*-2 than in position *sn*-1.

A higher level of 20:5n-3 was present in the *sn*-1 position of PG from the *Vibrio* grown at 5°C (5.6%) than in that of the same phospholipid of bacteria grown at 20°C (1.2%). The level of *trans* 16:1n-7 in position *sn*-1 was also higher at 5 than 20°C (4.2 vs. 1.6%). The fatty acid profile of position *sn*-2 was markedly influenced by growth temperature. In PG of bacteria grown at 5°C, *cis* 16:1n-7 accounted for 24.8% of the fatty acids in position *sn*-2, and this proportion increased to 43.6% at 20°C. In contrast, the content of 20:5n-3 decreased from 29.9% at 5°C to 8.2% at 20°C. The level of 17:1n-8 in position *sn*-2 also increased significantly with increasing growth temperature, and the overall content of monounsaturated fatty acids in this position was substantially higher at 20 than at 5°C. The level of *trans* 16:1n-7 in position *sn*-2 was not significantly influenced by growth temperature. Overall, the temperature-related changes in fatty acid composition of PG were more obvious in position *sn*-2 than in position *sn*-1.

DISCUSSION

It is well established that position *sn*-1 of bacterial phospholipids contains predominantly saturated fatty acids, whereas the *sn*-2 position is enriched in monounsaturated fatty acids (3,5). The data obtained in the present study confirm this pattern and further establish the unsaturated nature of position *sn*-2 by demonstrating that 20:5n-3 is located mainly in this position in both PE and PG in a *Vibrio* species of bacterium containing this PUFA. This is in keeping with the positional distribution patterns observed in eukaryotic phospholipids, where position *sn*-2 generally contains the highest proportion of PUFA (10,11), but is in contrast to the situation in cyanobacteria where C_{18} PUFA are concentrated in position *sn*-1 (12). It is likely that the preferential location of PUFA in the *sn*-2 position of bacterial phospholipids extends to 22:6n-3 in those species of bacteria which can produce this PUFA (16), although this remains to be established.

The finding that most of the *trans* 16:1n-7 in PE of the species of bacterium analyzed here is esterified to the *sn*-2 position of the glycerol moiety is in agreement with a previous

TABLE 2
Effect of Growth Temperature on Positional Distribution of Fatty Acids in Phosphatidylglycerol of *Vibrio* sp.[a]

	5°C			20°C		
Fatty acids	Overall composition	Position *sn*-1	Position *sn*-2	Overall composition	Position *sn*-1	Position *sn*-2
i-13:0	—	—	—	—	0.1 ± 0.1	1.2 ± 0.8
13:0	0.8 ± 0.4	0.4 ± 0.4	0.4 ± 0.4	0.3 ± 0 3	—	—
i-14:0	—	—	—	—	—	0.1 ± 0.1
14:0	1.5 ± 0.4[d]	0.9± 1.2	0.6 ± 0.3	0.5 ± 0.3	0.8 ± 0.4	0 6 ± 0 2
14:1n-5	—	—	—	—	—	0.1 ± 0.1
i-15:0	3.9 ± 0.5	4.2 ± 4.5	1.3 ± 0.7[f]	4.6 ± 1.1	5.7 ± 2.8	4.2 ± 0.5
a-15:0	—	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	—
15:0	3.2 ± 0.5	3.3 ± 2.7	0.8 ± 0.1[e]	3.0 ± 0.3	4.0 ± 1.0	0.5 ± 0.1
15:1n-6	1.4 ± 0.1	0.3 ± 0 4[c]	0.8 ± 0.5	1.9 ± 1.1	0.4 ± 0.0	1.3 ± 0.2
16:0	14.8 ± 1.3	23.5 ± 1.3	10.7 ± 3.3[d]	15.0 ± 1.1	25.3 ± 1.3	3.6 ± 1.3
16:1n-9	1.7 ± 0.2	1.5 ± 0.4	1.3 ± 0.1[f]	1.2 ± 0.6	1.6 ± 0.1	2.7 ± 0.1
cis 16:1n-7	25.2 ± 1.0[f]	11.7 ± 4.0	24.8 ± 5.5[f]	33.3 ± 3.0	13.3 ± 1.4	43.6 ± 4.1
trans 16:1n-7	7.0 ± 1.7[f]	4.2 ± 1.2[d]	8.0 ± 3.3	1.7 ± 0.5	1.6 ± 0.6	5.8 ± 1.3
17:0	1.0 ± 0.2[f]	2.3 ± 0.6[f]	0.5 ± 0.2[e]	2.2 ± 0.2	5.1 ± 1.0	0.2 ± 0.1
17:1n-8	7.7 ± 1.6[f]	9.2 ± 1.2	7.5 ± 1.1[f]	14.0 ± 1.4	10.4 ± 1.0	16.4 ± 1.5
17:1n-6	1.2 ± 0.1	1.6 ± 0.3	0.2 ± 0.1	1.1 ± 0.1	1.6 ± 0.2	0.2 ± 0.1
18:0	2.8 ± 0.7	8.2 ± 6.1	5.2 ± 1.8[d]	1.8 ± 0.9	3.7 ± 0.7	1.6 ± 0.7
18:1n-9	4.0 ± 0.9[d]	6.5 ± 3.3	5.9 ± 1.1[d]	7.2 ± 1.4	6.4 ± 0.7	8.0 ± 0.2
18:1n-7	5 4 ± 0.4[d]	15.7 ± 5.6	1.9 ± 0.4[e]	6.6 ± 0.6	13.9 ± 1.2	1.3 ± 0.1
20:5n-3	17.7 ± 1.3[f]	5.6 ± 1.6[f]	29.9 ± 6.4[f]	3.7 ± 2.2	1.2 ± 0.7	8.2 ± 1.0
Total sat.	28.0 ± 1.6	42.9 ± 4.3	19.7 ± 4.1[e]	27.5 ± 0.6	45.1 ± 5.1	12.4 ± 3.1
Total mono.	53.6 ± 1.2[f]	50.7 ± 4.0	50.4 ± 8.7[f]	67.0 ± 2.9	49.1 ± 4.1	79.4 ± 4.1
Total PUFA	17.7 ± 1.3[f]	5.6 ± 1.6[f]	29.8 ± 7.0[f]	3.7 ± 2.2	1.2 ± 0.7	8.2 ± 1.0
Unidentified	0.7 ± 0.2	0.8 ± 0.3	0.1 ± 0.1	1.8 ± 0.1	4.8 ± 0.4	—

[a]Values are mol% and are means ± SD of six cultures. Values at 5°C with superscripts are significantly different from the corresponding 20° value; [b]$P < 0.05$: [c]$P < 0.02$; [d]$P < 0.01$; [e]$P < 0.002$; [f]$P < 0.001$. *i-, iso; a-, anteiso;* sat., saturated fatty acids; mono., monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

study of the ABE-1 strain of *Vibrio,* which also contains this *trans*-monounsaturated fatty acid but no PUFA (8). In the species examined here, both *trans* 16:1n-7 and 20:5n-3 are located predominantly in the same position of PE with the level of the *trans* fatty acid exceeding that of the PUFA. It is notable that although the proportions of both fatty acids increased in position *sn*-2 with a reduction in growth temperature, the ratio of *trans* 16:1n-7 to 20:5n-3 was lower at 5°C (1.8) than at 20°C (2.6). This demonstrates that the magnitude of increase in 20:5n-3 in response to decreases in growth temperature is greater than that observed in the *trans* fatty acid.

The increase in the level of 20:5n-3 observed in bacterial phospholipids in response to a decreased growth temperature is in keeping with the frequently reported increases in the degree of unsaturation of phospholipids which occur during the adaptation of poikilothermic animals and plants to a reduced environmental temperature (1,2). However, in marked contrast to the situation observed here, the level of *trans* 16:1n-7 has been reported to be lower at 5°C than at 20°C in the *Vibrio* sp. strain ABE-1 (7,8). This difference in response between the two species of *Vibrio* may be related to differences in the other fatty acids present in PE. In comparison with the species of *Vibrio* examined here, *Vibrio* strain ABE-1 contains a more restricted pattern of fatty acids and, within its PE, changes in *trans* 16:1n-7 are reciprocated almost exclusively by changes in the level of *cis* 16:1n-7 (7,8). In contrast, the *Vibrio* in this study contains a wide range of fatty acids, and the levels of several fatty acids are influenced by growth temperature. In particular, it is notable that the increase in *trans* 16:1n-7 (11 mol%) observed when the bacteria were grown at 5°C compared to 20°C was matched by an almost equivalent decrease in the level of *iso* 15:0. Although the melting point and phase transition temperature of *iso*-15:0 are lower than those of saturated C_{14}–C_{18} straight chain fatty acids, the melting point of *iso* 15:0 (51.7°C) (5) is still higher than that of *trans* 16:1n-7 (32–33°C) (17). Consequently, it may be expected that an increase in the proportion of the latter fatty acid with a lowering of growth temperature would assist in maintaining membrane fluidity.

The observed positional distribution patterns refute our previous suggestion that a *trans* 16:1n-7/20:5n-3 molecular species of phospholipid is involved in temperature adaptation of membranes in this species of bacterium (9). Although the relative amounts of individual molecular species of PE which occurred at the two growth temperatures were not measured directly, it can be deduced from the positional distribution of fatty acids that the principal molecular species of PE at 20°C probably contained 16:0 or *iso*-15:0 in the *sn*-1 position coupled with *iso*-15:0 or *cis* 16:1 n-7 at position *sn*-2. At 5°C, higher proportions of molecular species containing *cis* 16:1n-7 at position *sn*-1 and *trans* 16:1n-7 or 20:5n-3 at position *sn*-2 would have been present. Thus, a *trans*

16:1n-7/20:5n-3 molecular species, if present, would only have been a minor molecular species at both temperatures.

The positional distribution of *trans* fatty acids in PG of bacteria has not been previously reported. Although the present results demonstrate that position *sn*-2 of PG contained more *trans* 16:1n-7 than position *sn*-1, as in PE, the difference between the two positions was not so great as observed with PE. At the same time, the position *sn*-2 of PG was much richer in 20:5n-3 than the same position of PE, and the proportion of 20:5n-3 always exceeded that of *trans* 16:1n-7 regardless of temperature. Because PE and PG both arise from CDP-diacylglycerol in Gram negative bacteria, similarities in fatty acid composition might be expected. However, PG is known to be a highly active substrate in the lipid metabolism of bacteria and has more potential than PE for changing its fatty acid composition (3).

The positional data suggest that the biggest temperature-induced changes in molecular species composition of PG involve those containing 20:5n-3 rather than *trans* 16:1n-7. Thus, at 20°C molecular species of PG containing 16:0 or a *cis* monounsaturated fatty acid coupled with *cis* 16:1n-7 at position *sn*-2 are major molecular species, while a decrease in growth temperature results in an increase in molecular species with 20:5n-3 at position *sn*-2. At the same time, however, small amounts of di-20:5n-3 molecular species may also be formed. Analysis by HPLC of the molecular species of PE and PG is of obvious interest in elucidating thermal adaptation effects in this *Vibrio* species but presents a challenge on account of the constituent levels of odd-chain and branched fatty acids.

In *Vibrio* sp. strain ABE-1, the increase in *trans* 16:1n-7 observed during adaptation to growth at a high temperature is matched closely by the decrease in *cis* 16:1n-7, and the conversion of *cis* to *trans* isomers by an isomerase has been demonstrated when the bacterium adapts to growth at higher temperature in the presence of inhibitors of *de novo* fatty acid synthesis (7,8). However, it is notable that, in the *Vibrio* species studied here, the levels of *cis* and *trans* 16:1n-7 show no inverse relationship in either PE or PG during temperature adaptation, and it remains to be established whether the bacterium can adopt the isomerization of preexisting *cis* 16:1n-7 to its *trans* isomer as a strategy for adaptation to growth at low temperature.

It has been recently shown that, in the phospholipids of some species of lower fungi, n-3 PUFA are located predominantly in position *sn*-1, whereas n-6 PUFA mainly occupy position *sn*-2 (18). This specific positional distribution has been attributed to the Δ15 desaturase having a specificity for the acyl group in position *sn*-1. Although the preferential esterification of 20:5n-3 in position *sn*-2 of *Vibrio* suggests that such a positional specificity of desaturases does not operate in this bacterium, very little is known of the mechanisms of PUFA biosynthesis in bacteria. It remains to be established whether intact phospholipids, as in fungi, plants and protozoa, or acyl thioesters as in animals, serve as substrates for desaturase enzymes in bacteria.

REFERENCES

1. Harwood, J.L. (1994) *Prog. Lipid Res. 33,* 193–202.
2. Hazel, J.R., and Williams, E.E. (1990) *Prog. Lipid Res. 29,* 167–227.
3. Harwood, J.L., and Russell, N.J. (1984) *Lipids in Plants and Microbes,* pp. 7–18, George Allen and Unwin, London.
4. Bhakoo, M., and Herbert, R. (1979) *Arch. Microbiol. 121,* 121–127.
5. Kaneda, T. (1991) *Microbiol. Rev. 55,* 288–302.
6. Suutari, M., and Laakso, S. (1992) *Biochim. Biophys. Acta 1126,* 119–124.
7. Okuyama, H., Sasaki, S., Higashi, S., and Murata, N. (1990) *J. Bacteriol. 172,* 3515–3518.
8. Okuyama, H., Okajima, N., Sasaki, S., Higashi, S., and Murata, N. (1991) *Biochim. Biophys. Acta 1084,* 13–20.
9. Henderson, R.J., Millar, R.-M., Sargent, J.R., and Jostensen, J.-P. (1993) *Lipids 28,* 389–396.
10. Van Deenen, L.L.M. (1965) *Prog. Chem. Fats Other Lipids 8,* 1–127.
11. Hazel, J.R., and Landrey, S.R. (1988) *Am. J. Physiol. 255,* R628–R634.
12. Murata, N. (1989) *J. Bioenerg. Biomembranes 21,* 61–75.
13. Jostensen, J.-P., Olsen, R.E., and Ringo, E. (1990) *Fisk. Dir. Skr. Ser. Ernaering* 3, 31–35.
14. Olsen, R.E., and Henderson, R.J. (1989) *J. Exp. Mar. Biol. Ecol. 129,* 189–197.
15. Christie, W.W. (1989) *Gas Chromatography and Lipids,* pp. 66–128, The Oily Press Ltd., Dundee.
16. Yano, Y., Nakayama, A., Saito, H., and Ishihara, K. (1994) *Lipids 29,* 527–528.
17. Gunstone, F.D., Harwood, J.L., and Padley, F.B. (1994) *The Lipid Handbook*, 2nd edn., p. 187, Chapman and Hall, London.
18. Kendrick, A., and Ratledge, C. (1992) *Lipids 27,* 505–508.

[Received August 31, 1994, and in Revised form December 20, 1994; Revision accepted December 27, 1994]

ANNOUNCEMENTS

1995

June

2nd International Congress of the International Society for the Study of Fatty Acids and Lipids, June 7–10, National Institutes of Health, Bethesda, Maryland, USA. Information: A.P. Simopolous, ISSFAL, 2001 S St., N.W., Suite 530, Washington, DC 20009 USA.

18th Nordic Lipid Symposium, June 18–21, Reykjavik, Iceland. Information: G. Lambertsen, Lipidforum Secretariat, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-325724).

August

36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Beijing, China, Oct. 7–11, 1995. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

Regulation of Biosynthesis and Function of Isopentenoids: An Introduction

W. David Nes[a,*] and Edward J. Parish[b]

[a]Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas and [b]Department of Chemistry, Auburn University, Auburn, Alabama

During the AOCS Annual Meeting & Expo at the Atlanta Marriott Marquis in Atlanta, Georgia, which took place May 8–12, 1994, a two-day symposium was held on the "Regulation of Biosynthesis and Function of Isopentenoids." The topics ranged from studies on molecular biology, pathway sequencing and regulation of sterol biosynthesis and function in plants, animals and bacteria, to conformational analyses and the synthesis of biorationally designed, mechanism-based inactivators of sterol production. The four sessions on isopentenoids, including sterols and sterol-like structures, involved more than a dozen invited plenary lectures by experts from academia, industry, and federal laboratories [*INFORM* 5:469–470, 485–486, 493–494, 508–509 (1994)].

This was the ninth AOCS-sponsored symposium held on the topic; the first was organized by the late Henry Kircher and was held during the 1970 AOCS Annual Meeting & Expo in New Orleans. The proceedings of one of these meetings were published as an AOCS monograph in 1991, and six of the symposia were published as units of peer-reviewed papers in *Lipids*. The level of participation over the years at these meetings has been most impressive, and many of the symposia papers that appeared in *Lipids* are frequently cited in the scientific literature. The present issue of *Lipids* contains eleven papers based on Atlanta symposium presentations. They include seven reviews and four original research articles.

We wish to thank Wolfgang J. Baumann, then Editor-in-Chief of *Lipids*, for his special efforts in peer-reviewing and editing the papers and for his willingness to publish this symposium as a unit publication in *Lipids*. His personal attention to each of the papers of this and of the earlier symposia is much appreciated. Finally, we also wish to thank AOCS, Dupont, Marion Merrell Dow, and Johnson & Johnson for their support.

*To whom correspondence should be addressed at Department of Chemistry and Biochemistry, Box 4260, Texas Tech University, Lubbock, TX 79409-1061.

Copyright © 1995 by AOCS Press

Some New Aspects of Isoprenoid Biosynthesis in Plants—A Review[1]

Thomas J. Bach*

C.N.R.S.-I.B.M.P., Département d'Enzymologie Cellulaire et Moléculaire, Institut de Botanique, Université Louis Pasteur, F-67083 Strasbourg, France

ABSTRACT: Plants are capable of synthesizing a myriad of isoprenoids and prenyl lipids. Much attention has been focused on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the enzyme that synthesizes mevalonate and is generally considered responsible for the regulation of substrate flux to isoprenoids. In contrast to vertebrates, where there seems to exist only one HMGR gene, in plants a small family of isogenes appears differentially expressed in regard to location and time. Much less is known in plants about the preceding steps, *viz.* the conversion of acetyl-CoA to HMG–CoA. An enzyme system has been isolated from radish that can catalyze this transformation, and which shows some unusual properties *in vitro*. The intracellular localization of the early steps of isoprenoid biosynthesis in plant cells is still a matter of debate. The various observations and hypotheses derived from incorporation and inhibition studies are somewhat contradictory, and an attempt is being made to rationalize various findings that do not at first seem compatible. There are good arguments in favor of an exclusively cytoplasmic formation of isopentenyl pyrophosphate (IPP) via mevalonic acid, but other studies and observations suggest an independent formation in plastids. Other possibilities are being considered, such as the existence of independent (compartmentalized) biosynthetic pathways of IPP formation *via* the so-called Rohmer pathway. Substrate channeling through the formation of end product-specific multienzyme complexes (metabolons) with no release of substrate intermediates will also be discussed.

Lipids 30, 191–202 (1995).

A myriad of natural products are derivatives of a common C_5 isoprene unit and are thus classified as isoprenoids. As it is impossible to cover the broad field of plant isoprenoid biosynthesis, I will focus on some recent aspects, which, at first sight, seem incompatible or even in conflict with each other, and may be clarified by being looked at from a new angle. The intracellular localization of enzymes, especially of those at the entry of the multibranched isoprenoid pathway, is still a matter of debate and will be discussed in light of new findings. Recently, evidence also has been presented for the existence of alternate pathways of mevalonate and/or isopentenyl pyrophosphate (IPP) formation in plants, the latter one being reminiscent of the so-called Rohmer pathway that is operative in certain bacteria. The possible existence of two independent and strictly compartmentalized biosynthetic routes for the formation of a single intermediate would explain some contradictory results that have been obtained and that suggested the partial or complete segregation of organelles from cytoplasmic enzymes, as was deduced from incorporation and inhibition studies as well as through determination of enzyme activities in subcellular fractions. Moreover, such an alternate pathway would explain the obviously close relationship between isoprene production and the Calvin cycle in certain plants, as well as the unequal labeling of certain isoprenoids from various precursors.

GENERAL IMPORTANCE OF THE MULTIBRANCHED ISOPRENOID PATHWAY IN PLANTS

To date, some 22,000 isoprenoids (terpenoids, isopentenoids) have been characterized (1), most being of plant origin. For instance, from tobacco alone, more than 470 isoprenoid derivatives had been identified by 1985 (2). It is apparent that the multibranched isoprenoid pathway in plants represents one of the most complicated biosynthetic pathways known (Fig. 1). There exists hardly a metabolic process in plants that does not require the biochemical or physiological involvement of isoprenoids, be it "pure" isoprenoids consisting only of isoprene units, or "mixed" isoprenoids, with only part of the molecule being derived *via* the isoprenoid pathway. Besides their function in photosynthesis (carotenoids, chlorophylls, phylloquinone, plastoquinone), respiration (ubiquinone, cytochrome a), membrane architecture (sterols and triterpenoids), regulation of growth and development (gibberellic acids, abscisic acid, brassinosteroids, certain cytokinins), defense against pathogen attack (numerous isoprenoid phytoalexins), exchange of signals and so forth, prenylation of proteins might be thought of as one important

[1]Based in part on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at the C.N.R.S., Institut de Biologie Moléculaire des Plantes, Département d'Enzymologie Cellulaire et Moléculaire, 28 rue Goethe, F-67083 Strasbourg, Cédex, France.

Abbreviations: AACT, acetoacetyl-CoA thiolase; ALS, acetolactate synthase; CAL, chalcomoracin; DMAPP, dimethylallyl pyrophosphate; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; HMGL, HMG–CoA lyase; HMGR, HMG–CoA reductase; HMGS, HMG–CoA synthase; IPP, isopentenyl pyrophosphate; MVA, mevalonic acid; MVAP, mevalonate phosphate; MVAPP, mevalonate pyrophosphate; PDC, pyruvate decarboxylase; PP, pyrophosphate; PQQ, pyrroloquinoline quinone; SRP, signal recognition particle; TPP, thiamine diphosphate.

Copyright © 1995 by AOCS Press

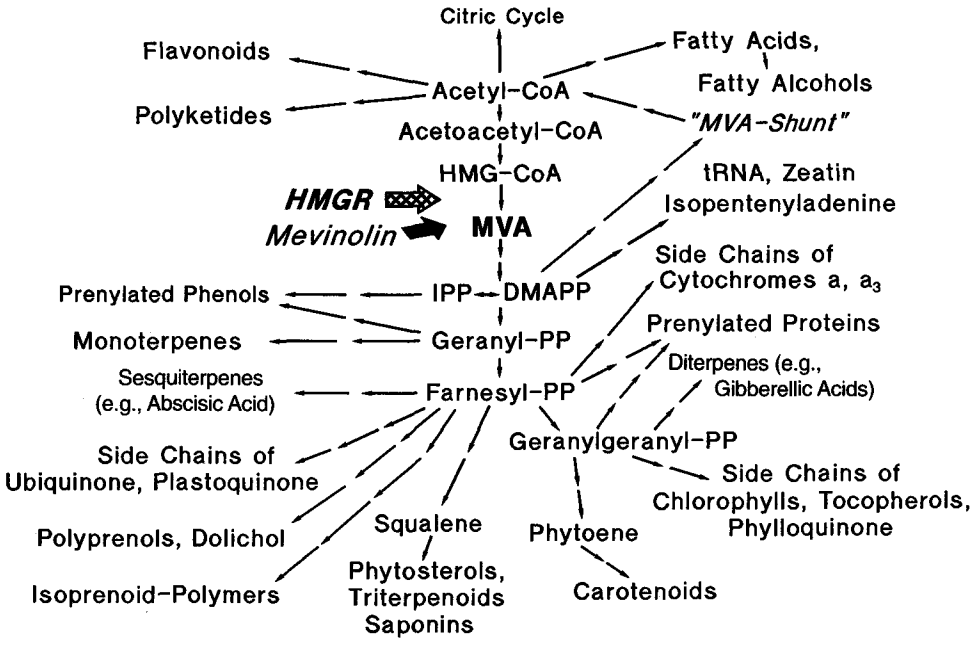


FIG. 1. The multibranched isoprenoid pathway in plants. The position of the key enzyme 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase and its specific inhibitor mevinolin is indicated. MVA, mevalonic acid; HMGR, HMG–CoA reductase; IPP, isopentenyl pyrophosphate; PP, pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

mechanism of membrane targeting and cell cycle regulation. However, in view of the new findings discussed below, this "classical" scheme may need some modification.

Mevalonic acid (MVA) as specific precursor of isoprenoids. With the discovery of MVA isolated from distillates of brewers yeast as an acetate-replacing growth factor in the culture broth of *Lactobacillus acidophilus* (3), and with its excellent incorporation into cholesterol in rat liver homogenates (4,5), the "true" committed precursor for isoprenoid biosynthesis has been identified. By monitoring the incorporation of labeled MVA into various plant isoprenoids (see Refs. 6–10, and references therein) the principle that *(R)*-MVA serves as precursor of isoprenoids became accepted. The degree of radiolabeling from MVA found in isoprenoids other than sterols or sterol precursors, such as squalene, cycloartenol etc., can be rather negligible (Refs. 7, 10, 11, and literature cited therein). The radiochemical purity of the MVA-derived, radiolabeled compound has not been proven in every case. This also holds true for the incorporation of acetate (or of precursors that can be easily metabolized to acetate) into isoprenoids. Since this substrate serves as a precursor in various syntheses, including the biosynthesis of fatty acids and their derivatives, contamination by other lipophilic compounds can cause serious problems. For example, long-chain fatty alcohols are known to migrate on thin-layer chromatography plates with R_f values similar to that of sterols (12), and complete purification of sterols requires several steps of column chromatography and repeated recrystallization. Thus, the radiochemical purity of the radiolabeled products of the multibranched isoprenoid pathway was not really proven in each case, nor had the time of exposure to radiolabeled precursors such as ^{14}C- or ^{3}H-labeled MVA excluded metabolic degradation and, consequently, reuse of breakdown products, e.g., of acetate, in the *de novo* biosynthesis of isoprenoids. Further, as already discussed by Banthorpe *et al.* (7) and by Goad (10), the unavoidable introduction of unphysiologically large quantities of MVA might result in the degradation of the additive to products that may also act as enzyme inhibitors or lead to the induction of salvage degradation pathways. On the other hand, a poor incorporation of MVA, due to its limited uptake and transport, does not prove *per se* that it is not an obligate intermediate in the biosynthesis of a certain isoprenoid.

Properties of plant R-*mevalonate: NADP$^+$ oxidoreductase, CoA-acylating, EC 1.1.1.34 (3-hydroxy-3-methylglutaryl–CoA; HMGR).* It is widely accepted that HMGR, catalyzing the NADPH-dependent double-reduction of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) to MVA, plays a crucial role in the part of the isoprenoid pathway leading to phytosterols (13–16). Treatment of seedlings with mevinolin (lovastatin), a natural antibiotic and HMGR inhibitor (17–21), as well by its natural and artificial analogues leads to severe disturbances in plant growth (16,18,22,23–25), due to the specific block of *de novo* sterol biosynthesis. A further argument in favor of a regulatory role of HMGR in phytosterol accumulation was derived from the observation that in triazole-resistant tobacco mutant cells overproducing sterols (26), HMGR activity had increased 3- to 4-fold (27). Overproduced sterols, largely in the form of biosynthetic precursors, such as cycloartenol, appear as fatty acyl esters in lipid

droplets of the cytoplasm, but not in membranes (28,29). The same phenotype is present in transformed tobacco cells overexpressing the soluble part of hamster HMGR (30) or overexpressing rubber tree HMGR (H. Schaller, M.-L. Chye, and P. Benveniste, personal communication). Apparently, plants derived from tobacco mutants or from transformed cells overexpressing HMGR genes exhibit a normal phenotype and growth rate, without significant changes in pigment composition. Furthermore, feeding of exogenous MVA to alfalfa seedlings and cell cultures (31) led to the accumulation of phytosterol intermediates, further underlining the central regulatory role of HMGR in the control of substrate flux from acetyl-CoA to phytosterols as discussed earlier (13).

Hydrophobicity plots according to Kyte and Doolittle (32) predict that all plant HMGR isozymes sequenced so far (33–48) should contain a highly conserved membrane domain consisting of two hydrophobic amino acid sequences, separated from the soluble and highly conserved domain bearing the catalytic site by a linker region without any significant homology (Refs. 49,50). In plants it appears that HMGR isozymes are encoded by a small family of genes, and evidence is emerging that these genes and gene products are placed under developmental or environmental control by factors such as light, xenobiotics, or microbial elicitors (41,43, 51–66; see Refs. 16,53,66–68 for additional references), somehow reflecting the greater diversity of mevalonate derivatives in the realm of plants.

In contrast to the sequence areas coding for the sites of catalytic activity, found to be highly conserved in all eukaryotic HMGR enzymes (see Refs. 59,69, and literature cited therein), the regions of the membrane anchor and especially the neighboring linker are characterized by evolutionary sequence diversity. Animal and yeast HMGR types contain eight such hydrophobic regions in the membrane anchor (70,71). When two radish HMGR isogenes (cDNA derived from *HMG1* and *HMG2*) were expressed in an MVA-auxotrophic yeast mutant, however, we could show that the two membrane-spanning amino acid sequences are sufficient to direct the targeting of nascent radish HMGR into yeast membranes. Western blot analyses revealed that, in the heterologous system, proteins of the correct size (about 63 kDa) had been integrated into the membrane(s) (for details, see Ref. 72). It must be noted, however, that there exists a considerable controversy as to the exact intracellular localization of HMGR in plants (see Refs. 11,14,16,50,51,53,73–78, and literature cited therein). As known so far, all plant HMGR sequences are characterized by the absence of a typical leader peptide indicative of a translocation from the cytoplasm into organelles. Recent *in vitro* transcription/translation experiments with two rather divergent isogenes encoding *Arabidopsis* HMGR showed a co-translational insertion of plant HMGR protein into endoplasmic reticulum-derived microsomes (50), similar to the situation in vertebrate cells. However, for the import of proteins destined for the outer chloroplast envelope, apparently no ATP-hydrolysis, no transit sequence, and no protease-sensitive envelope surface components are needed (79). Thus, a localization of an isozyme of HMGR in the outer plastid envelope as suggested by Wilson and Russell (63) cannot be ruled out. Boronat *et al.* (80) have recently reported the use of alternative promoters in the *Arabidopsis HMG1* gene, which results in the synthesis of two HMGR1 forms that differ in their *N*-terminal end. However, in an *in vitro* transcription/translation system, all forms of *Arabidopsis* HMGR isozymes were equally incorporated into microsomes, as in an SRP-dependent manner (N. Campos, and A. Boronat, personal communication). In tomato, where four HMGR isogenes have been identified which show differences in their temporal expression programs and inducibility by arachidonic acid, synthesis of MVA is essential for the development of green fruits (cell division and expansion), but is also linked to the conversion of chloroplasts to chromoplasts in parallel with lycopene accumulation (81; W. Gruissem, personal communication). Different HMGR isozymes might therefore direct the synthesis of different end products of the isoprenoid pathway. .

Conversion of acetyl-CoA to HMG–CoA. Following the pioneering work of Rudney and Lynen and their associates with yeast and mammalian tissue, it has been well documented (*cf.* Ref. 16, and literature cited therein) that the conversion of three units of acetyl-CoA to HMG–CoA is catalyzed by two distinct enzymes: (i) AACT (acetyl-CoA acetyltransferase, EC 2.1.3.9) catalyzing a Claisen-type condensation, and (ii) HMGS (*S*-3-hydroxy-3-methylglutaryl-coenzyme A:acetoacetyl-coenzyme A lyase, CoA acylating, EC 4.1.3.5), catalyzing an aldol condensation. Little was known until recently as to the situation in plants (11,14–16). We have purified and characterized a membrane-associated enzyme system from four-day-old etiolated radish seedlings (*Raphanus sativus* L.), capable of converting acetyl-CoA to HMG–CoA (82). This enzyme system, apparently catalyzing both partial reactions, is being referred to as AACT/HMG–CoA synthase (HMGS). The quite unusual *in vitro* properties of this enzyme system led us to believe that plants might have developed an additional mechanism of HMG–CoA synthesis different from that of other eukaryotes. The most puzzling feature of radish AACT/HMGS is its apparent activation by redox-cycling as mediated by the artificial redox couple Fe(II)/pyrroloquinoline quinone (PQQ), or alternatively by Fe(II) and higher concentrations of H_2O_2, suggesting the involvement of hydroxyl radicals formed by a Fenton-Haber-Weiss reaction [$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$]. This effect can be seen with crude solubilized radish AACT/HMGS, but it is more pronounced with purified enzyme and it can be further enhanced by addition of PQQ (83). It is evident that during the purification we remove a heat-stable cofactor system (that remains yet to be identified), which cannot only partially replace the PQQ but also, to a certain extent, Fe^{2+}. However, at present, we cannot exclude that this new system represents a multifunctional enzyme or even an intrinsic side-activity of a plant-specific enzyme, which might be less specific than we thought earlier. Although on sodium dodecyl sulfate gels we detected only a prominent band of about 56 kDa (82), in good agree-

ment with values obtained by gel filtration on various materials (16,42), an underlying impurity cannot yet be excluded. Conclusive proof of the existence of the so-called AACT/HMGS enzyme system will need to await the isolation of the respective genes. However, we have been able to complement a yeast *erg*-mutant, deficient in temperature-stable AACT with a radish cDNA and have thus isolated a gene that encodes a soluble form of AACT with a molecular weight of about 42 kDa (84; EMBL accession number X78116). This latter observation would support the conclusions by van der Heijden *et al.* (85,86), although their purification scheme, using cell-free preparations of *Catharanthus roseus* cell cultures, has apparently not yet resulted in a separation of AACT and HMGS activities. A method for the simultaneous determination of all intermediates of the isoprenoid pathway, starting with acetyl-CoA, has recently been developed (87). While all other intermediates could be found, depending on the substrate fed, in no case was free acetoacetyl-CoA detected in isolated cells capable of synthesizing monoterpenes. In our test system for AACT/HMGS, we could not trap free acetoacetyl-CoA as a putative intermediate (16). Thus, the situation is still far from being clear. Furthermore, the exact intracellular localization of HMG–CoA synthesis (i.e., cytoplasm vs. plastids and/or mitochondria) remains obscure unless corresponding genes have been cloned.

BRANCHED-CHAIN AMINO ACIDS AS PRECURSORS OF PLANT ISOPRENOIDS

L-Leucine and L-valine, when fed to germinating pea seeds (*Pisum sativum*) were incorporated into squalene and β-amyrin (88). Chemical degradation by ozonolysis of the radioactive squalene revealed an equal distribution of the radioactivity in the IPP-derived (four portions per molecule) and the dimethylallyl pyrophosphate (DMAPP)-derived moieties (two portions per molecule). However, an unbalanced distribution in favor of the DMAPP-derived moiety of monoterpenoids was found after feeding radioisotopically labeled L-leucine, L-valine, DL-alanine, acetate, and *R,S*-MVA to intact plants of *Cinnamomum camphora* and of *Pelargonium roseum* (89). The relatively low incorporation of amino acids was explained in several ways: (i) the radioactivity of amino acids is scattered into metabolites other than monoterpenoids; (ii) there is a substantial pool of amino acids which leads to dilution of radioactivity; and (iii) there is little permeation of the amino acids into the biosynthetic site of monoterpenoids (90). The DMAPP-derived moiety of monoterpenoids, biosynthesized from [*U*-^{14}C]leucine and [*U*-^{14}C]valine was labeled with more than 64% of the incorporated tracers, while this moiety when derived from [2-^{14}C]MVA contained less than 32% of the tracers (89). This lends support to the interpretation that both amino acids are incorporated not *via* MVA, but by an alternate route, e.g., a combination of the well known mammalian leucine degradation pathway and a reversal of the MVA shunt (see below). The distribution pattern in monoterpenoids after administration of [2-^{14}C]alanine was similar to that after incorporation of MVA, which indicates that alanine is first metabolized to acetyl-CoA, which then constructs preferentially the IPP-derived moiety of the monoterpenoids *via* MVA (89).

Contradictory results were obtained by Anastasis *et al.* (90) when they studied the incorporation of [2-^{13}C]- and [3-^{13}C]-leucines into sesquiterpenoid paniculides by tissue cultures of *Andrographis paniculata*. Apparently, HMG–CoA from leucine breakdown was not incorporated intact but was broken down to acetyl-CoA and subsequently incorporated *via* HMG–CoA and MVA. The same seems to hold true for phytosterols labeled under the same conditions (91). However, the incorporation time was 7 d, which might result in degradation of precursors and products, while in the studies summarized by Suga *et al.* (88) and Tange (89), incorporation times ranged from 12 to 72 h. From the incorporation pattern in bakuchiol, a meroterpene from *Psoralea corylifolia*, derived from leucine, valine, and isovaleric acid, it was concluded that these substrates are metabolized to CO_2, which is then incorporated (92).

Evidence for the occurrence of a MVA shunt pathway in plants. Evidence has been presented for the occurrence of a mevalonate shunt pathway in higher plants (12), with HMG–CoA as an intermediate, as it is most likely true for the degradation of leucine. Given the fact that the membrane fraction used to isolate AACT/HMGS from radish is also a major source for HMG–CoA lyase (HMGL) (EC 4.1.3.4), a key enzyme of the MVA shunt (93), its presence could indicate that in plants AACT/HMGS plays a role in a ketogenic cycle. However, to our knowledge, there is no clear information as to the metabolic fate in plants of acetoacetate generated by the HMG–CoA lyase (HMGL) reaction. However, though the activity of 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) has been demonstrated in plants (94–98), and also that of *trans*-3-methylglutaconyl-CoA hydratase (3-hydroxy-3-methylglutaryl-CoA hydrolyase, EC 4.2.1.18) (85,86), further evidence of an alternative peroxisomal pathway of leucine degradation has been presented by Gerbling (99), including free acids rather than CoA esters. At present the interdependence of those intervening and/or scavenging pathways is not yet known, nor is their regulation.

ISOPRENE EMISSION BY PLANTS

Many plants produce considerable amounts of isoprene (see Ref. 100, and literature cited therein) which can cause air pollution problems in some areas. Isoprene emission from plants may account for half of the total nonmethane hydrocarbon emissions from the biosphere to the troposphere, thereby influencing the oxidative capacity and generating tropospheric ozone and carbon monoxide (see Ref. 101, for literature). Environmental stimuli or wounding affect the rate of synthesis (100,102). This high amount of isoprene, derived from the IPP isomer DMAPP (103), can hardly be explained by the quite limited substrate flow from acetyl-CoA to MVA, because cellular HMGR activity, at least from what has been

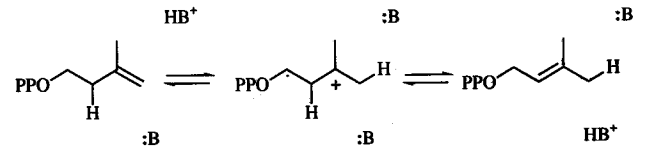


FIG. 2. Isopentenyl pyrophosphate isomerization to dimethylallyl pyrophosphate. The reaction proceeds *via* an acid-base catalyzed carbocationic mechanism. :B, basic amino acid residue involved in enzymatic catalysis; HB^+ protonated form (from Ref. 111). PPO, pyrophosphate esterified with hydroxyl groups of Δ^2- and Δ^3-isopentenol.

found in all plants studied so far, would not be sufficient to saturate such a rate of synthesis. Furthermore, it appears that there is a rather direct coupling to photosynthetic activity, *viz.* intermediates of the Calvin cycle (100,101,104–108). Most likely, pyruvate derived from 3-phosphoglyceric acid as the primary product of CO_2 fixation is a close precursor (see below for possible biosynthetic consequences). Isoprene is also a normal constituent of human breath (see Ref. 109, and literature cited therein), but in this case a clear coupling to the rate of cholesterol and mevalonate biosynthesis was shown. Blockade of MVA biosynthesis by the hypocholesterolemic drug mevinolin (lovastatin) leads to a decrease in cholesterol synthesis in liver, and to the same degree to a diminished production of isoprene (109).

Isoprene production might be explained by the enzymatic mechanism of IPP isomerization to DMAPP: Reardon and Abeles (110) and Muehlbacher and Poulter (111) have elegantly shown that IPP isomerase (isopentenyl-diphosphate Δ-isomerase, EC 5.3.3.2) catalyzes a reaction with an intermediate carbocation (Fig. 2). This isomerization involves an antarafacial transposition of a proton, suggesting that a conjugate acid-base pair works in tandem to catalyze the reaction (111). A similar carbocation intermediate occurs in the acid-catalyzed nonenzymatic conversion of DMAPP to isoprene in liver (112; Fig. 3). Elimination of diphosphate from DMAPP could result in the formation of a delocalized carbocation intermediate followed by the loss of a single proton,

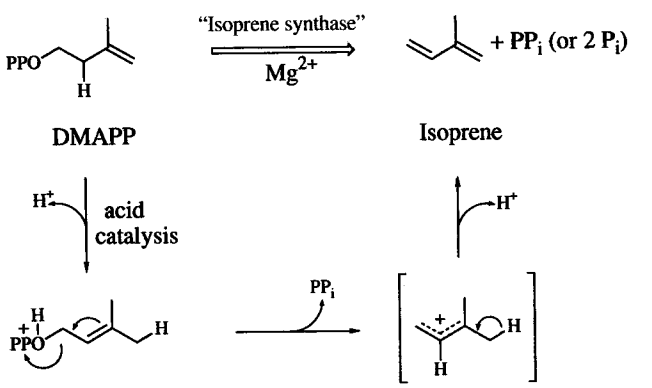


FIG. 3. The "isoprene synthase reaction." Comparison to the probable mechanism for the acid-catalyzed conversion of DMAPP to isoprene as proposed by Silver and Fall (103). The enzymatic and acid-catalyzed reactions may proceed *via* a delocalized carbocation intermediate (redrawn from Ref. 103). See Figures 1 and 2 for abbreviations.

which would yield isoprene (103). It might be speculated that the Mg^{2+}-dependent reaction is in fact identical to that catalyzed by the so-called isoprene synthase partially purified by Silver and Fall (103), and that the plant enzyme cannot closely control the isomerization, ultimately leading to an abortion of enzyme-bound substrate and hence to the production of isoprene (see Refs. 109,112).

THE ROHMER PATHWAY

Bacterial isoprenoid biosynthesis. Current biosynthetic evidence indicates that the steps from IPP to isoprenoids in Eubacteria are the same as those in eukaryotes (113–123; see Refs. 122–128 for further literature). The incorporation of ^{13}C-labeled precursors into ubiquinone-8 (123), and especially into prokaryotic hopanoids (128–131), sterol surrogates in bacterial membranes but also in the thylakoids of the cyanobacterium *Synechocystis* sp. (132), has revealed that the classical pathway of IPP formation starting from acetyl-CoA *via* acetoacetyl-CoA, HMG–CoA, MVA, mevalonate phosphate (MVAP), and mevalonate pyrophosphate (MVAPP) does not exist in a great variety of bacteria, including *Escherichia coli, Zymomonas mobilis, Methylobacterium organophilum* (133), and *Rhodopseudomonas palustris, R. acidophila, Acetobacter aceti* ssp. *xylinum* (134).

Zhou and White (123) argued that label from acetate could be incorporated after being metabolically "scrambled," i.e., *via* the glyoxylate cycle: The condensation of acetate (as acetyl-CoA) with glyoxylate by action of malate synthase (EC 4.1.3.2) would form malate, which could then be converted into pyruvate by malic enzyme (L-malate: $NADP^+$ oxidoreductase [oxalacetate-decarboxylating], EC 1.1.1.40). The incorporation of the C-3 and C-2 unit of the resulting pyruvate into IPP and hence into hopanoids is better explained by the model as proposed by Rohmer *et al.* (125; Fig. 4): The formation of the structure C-5 of IPP (better: of the structure "isoprene unit") would proceed in a way similar to the biosynthesis of L-valine. A TPP-activated acetaldehyde generated by pyruvate decarboxylation would be added to the C-2 carbonyl group of a dihydroxyacetone derivative (such as dihydroxyacetone, monohydroxyacetone, or their mono- or diphosphates, methylglyoxal, etc.), followed by a transposition reaction. The following reactions (reduction, isomerization, elimination of water) remain speculative, but the authors argue that they could again be similar to those found in L-valine biosynthesis. However, at least in the case of the ethanol-producing bacteria *Z. mobilis*, they could exclude L-valine or one of its precursors as substrate of IPP formation (see Ref. 125 for details).

An alternative IPP synthesis in plants akin to the bacterial Rohmer pathway? Hano *et al.* (135,136) have used callus and cell suspension cultures of *Morus alba* L. to study the biosynthesis of optically active prenylchalcones such as chalcomoracin. [2-^{13}C]MVA was not incorporated into the prenyl moiety of chalcomoracin, while it was incorporated into

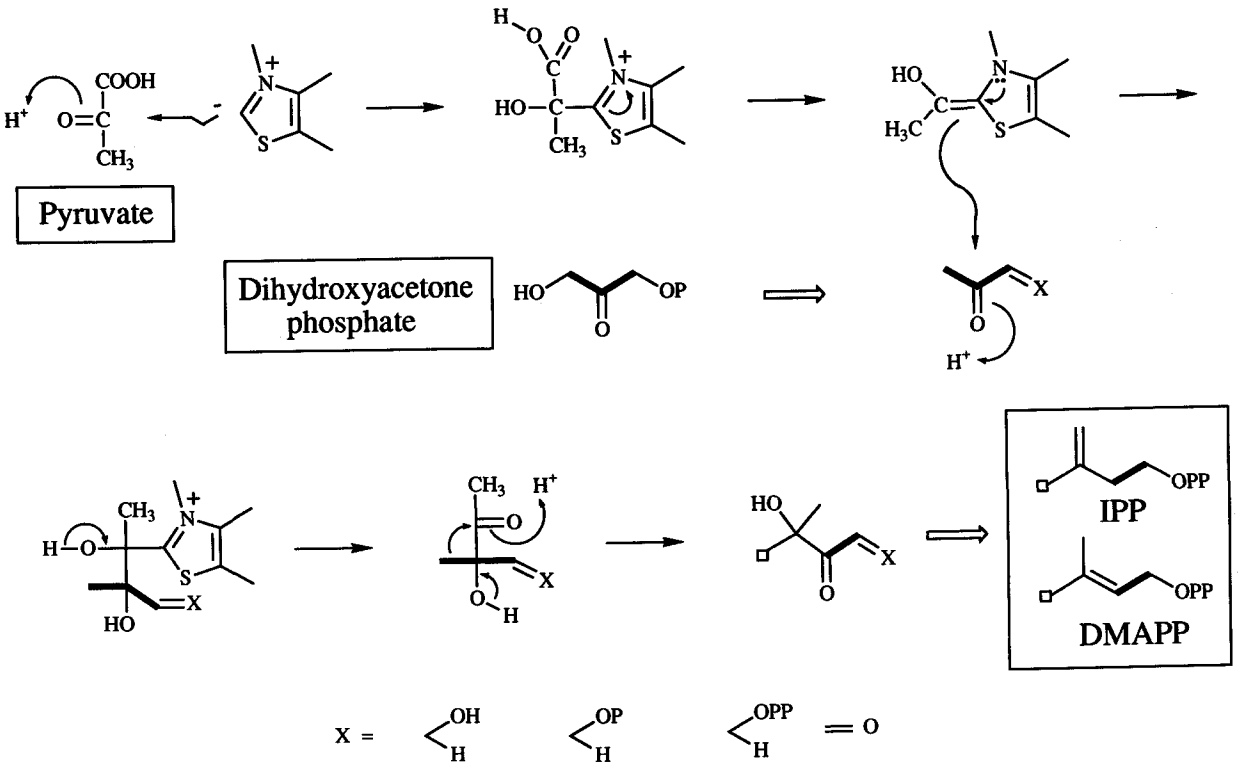


FIG. 4. The biosynthetic pathway of bacterial IPP formation as proposed by Rohmer *et al.* (125) (redrawn from Ref. 125). OPP, pyrophosphate esterified with hydroxyl group of Δ^2- and Δ^3-isopentenol. See Figure 1 for other abbreviations.

β-sitosterol. The labeling pattern of chalcomoracin from [2-^{13}C]-L-leucine was the same as from [1-^{13}C]acetate. Feeding D-[U-$^{13}C_6$]glucose, [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol (which formed [1,3,4,6-$^{13}C_4$]- and [2,5-$^{13}C_2$]glucose, respectively) demonstrated that of the three acetate units composing the hemiterpene moiety, the starter acetate unit is of glycolytic origin, whereas the second and third acetate units originate from the pentose phosphate cycle. Triose phosphates such as dihydroxyacetone phosphate and glyceraldehyde-3-phosphate are postulated to occupy the junction of this chimeric hemiterpene pathway with glycolysis and the pentose phosphate cycle (see Fig. 5). The major difference in the isotopic enrichments between the Rohmer pathway and the mechanism proposed by Hano *et al.* (135,136) is that ^{13}C-label coming from [1-^{13}C]acetate appears in C-4 of IPP, while the hemiterpene moieties of prenylchalcones were not enriched, and second in *M. alba* cell cultures, label from [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol labeled the C-1 and C-5 positions, or the C-2, C-3, and C-4 positions of IPP, respectively. Although the nuclear magnetic resonance data are quite conclusive, it is still difficult to rationalize in this model how acetyl-CoA units can be distinguished in the formation of HMG–CoA as an intermediate (135,136), if not assuming intermediates that are recognizable through tight binding to proteins/enzymes. This work needs to be complemented by (i) incorporation studies using specifically ^{13}C-labeled glucose (see below), and in particular (ii) the application of inhibitors of the mevinolin type, which should be capable of affecting the synthesis of chalcomoracin if the model is true.

In an independent study, presented at a previous isoprenoid meeting at Strasbourg/Bischenberg, Cartayrade *et al.* (137) compared the biosynthesis of β-sitosterol and of diterpenoid ginkgolides in embryos of *Ginkgo biloba*. (Ginkgolides act apparently as antagonists of platelet-activating factors and are thus of high pharmaceutical interest for the treatment of thrombosis, inflammatory reactions and cardiovascular disorders; for literature see Ref. 138.) In *G. biloba*, the IPP precursor of β-sitosterol is assembled *via* MVA formed by the classical pathway from acetyl-CoA *via* acetoacetyl-CoA, HMG–CoA. In contrast, the IPP units used for formation of ginkgolides are apparently assembled through decarboxylative condensation of pyruvate with a triose phosphate, similar or identical to the Rohmer pathway (137,139). Further, these two independent biosynthetic machineries are compartmentalized. However, there seems to exist some "leakage" at the C-15 level such that a small quantity of farnesyl-pyrophosphate (PP) made in the cytoplasm as for β-sitosterol synthesis, is linked then by action of geranylgeranyl-PP synthase to an IPP unit made by the alternative pathway, most possibly in the plastid (137,139). Soler *et al.* (140) presented evi-dence of an IPP translocator in the plastid envelope, but the K_m of about 500 μM with respect to IPP does not convincingly show that it is really a very specific protein. It might function as a (pyro)phosphate translocator that

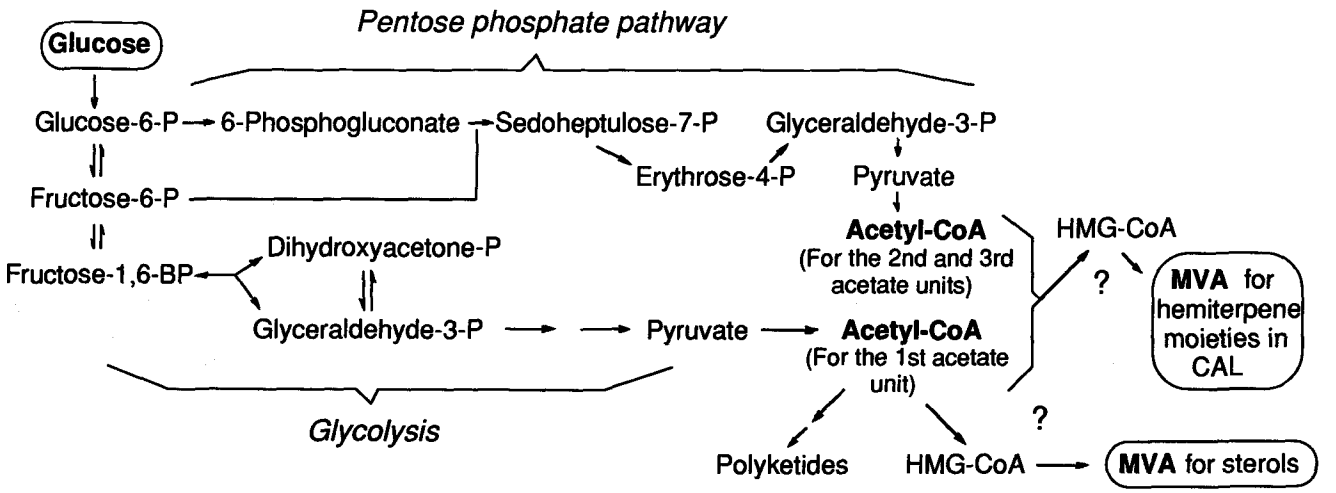


FIG. 5. Biosynthetic routes from glucose to the hemiterpene moiety of chalcomoracin (CAL) and to sterols in *Morus alba* cells as proposed by Hano *et al.* (135,136). For details, e.g., labeling patterns, see References 135,136. Note that differential mechanisms are proposed for the synthesis of HMG–CoA and of MVA used for CAL and sterols, respectively. Question marks denote uncertainties as to the enzyme systems involved (see text for discussion). See Figure 1 for other abbreviations.

could perhaps also accept higher IPP homologs such as farnesyl-PP.

Evidence of an enzymatic machinery for the Rohmer pathway? A crucial point in this new model of IPP formation in bacteria and eukaryotes as well is the fact that until now individual enzyme reactions have not yet been identified. An exception could be the initial reaction, the formation of thiamine diphosphate (TPP)-acetaldehyde, catalyzed by pyruvate decarboxylase (PDC, 2-oxoacid carboxylase, EC 4.1.1.1), an enzyme that is not present in animal tissues, but has been isolated from yeast (see Ref. 141, and literature cited therein) and also from some plants such as maize (142). Yeast PDC can also catalyze an aldol-type condensation reaction between two molecules of acetaldehyde to form the α-ketol acetoin in a concerted reaction mechanism (141). It seems reasonable to assume a similar reaction pathway for the reaction of the TPP-bound "acetaldehyde" [2-(1-hydroxyethyl)TPP] with the dihydroxyacetone derivative as postulated by Rohmer *et al.* (125). Given the fact that the hyperbolic kinetic properties and apparent molecular weights of the yeast (iso-)enzyme(s) (tetramers, native mass of 242 kDa) and the maize enzymes (kernels: 61 and 60 kDa subunits, roots: 59 and 58 kDa subunits), tetrameric structure for both isozymes (142) are so similar, then it would be no surprise if both enzymes could catalyze the same condensation reaction as well. However, PDC is a typical enzyme of anaerobic metabolism (see Ref. 143 and literature therein), and corresponding mRNA are induced anaerobically (144,145). It should be therefore difficult for acetaldehyde to "escape" the action of alcohol dehydrogenase (EC 1.1.1.1), which is also specifically enhanced under anaerobic conditions. Maize PDC, having a sharp pH optimum of 5.8, is inactive at the pH >7 of aerobically metabolizing tissues (142). However, as an alternative, it could be speculated that the entry reaction of the pyruvate dehydrogenase complex, pyruvate decarboxylation, which is mechanistically identical, could give rise to the same intermediate 2-(1-hydroxyethyl) derivative of the thiazol ring of TPP, which somehow would escape the subsequent dehydrogenation and the resulting esterification of the acetyl unit to coenzyme A. Plant cells contain two pyruvate dehydrogenase complexes, one in the mitochondria and the other in the plastids (see Ref. 146, and literature cited therein). If such a "side reaction" would exist, then incorporation data using pyruvate as a precursor of plastid isoprenoids and fatty acids (76,78,147) might be reinterpreted so that pyruvate decarboxylation could lead to a reactive intermediate exclusively used for IPP synthesis, while full conversion of pyruvate to acetyl-CoA would yield a pool that is exclusively used for fatty acid biosynthesis. In this way, the problem of two independent acetyl-CoA pools in the same subcellular compartment with little or no exchange, as postulated by Schultz and co-workers (76,78), could be easily circumvented.

In thiamine-deficient mutants of *Nicotiana sylvestris,* the synthesis of carotenes and chlorophylls, but not of fatty acids is blocked (148). Since thiamine (in the form of TPP) is a mandatory cofactor for the decarboxylation of pyruvate, the interruption of pigment synthesis in its absence might further indicate the tight linkage to the activity of the pyruvate dehydrogenase complex or to PDC. In this case the contribution of acetyl-CoA formed from pyruvate to fatty acid synthesis would be negligible as compared to acetate imported from outside of the chloroplast (148). Acetolactate synthase (ALS, EC 4.1.3.18, also commonly referred to as acetohydroxy acid synthase) is also capable of converting pyruvate to the abovementioned 2-(1-hydroxyethyl)-TPP intermediate (149). Competition between pyruvate and ketobutyrate for this intermediate defines the partitioning ratio with respect to the two physiological reactions, *viz.* one specific to isoleucine formation and the other specific to valine or leucine biosynthesis (149). The oxygenase side reaction of ALS is explained by the competition between molecular oxygen and pyruvate or ketobutyrate for the hydroxyethyl-TPP intermediate (149).

Although the fixed-time colorimetric assay for ALS employed by these authors would not distinguish between acetolactate and acetoin formation, it seems rather unlikely that an enzyme that is capable of closely controlling the partitioning ratio between the structural homologs pyruvate and ketobutyrate would accept further substrates for condensation, such as triosephosphates. Thiamine thiazolone pyrophosphate, a transition state analog of the reaction intermediate hydroxyethyl-TPP, has been shown to indiscriminately inhibit enzymes using a thiamine cofactor (150); however, for the study of entry reactions of the Rohmer pathway, such compounds might be useful as molecular probes.

CONSEQUENCES FOR THE SO-CALLED SEGREGATION MODEL OF ISOPRENOID BIOSYNTHESIS

Kleinig and his associates (11,151,152) have speculated on the presence of a synthetic pathway leading to IPP and not requiring the intermediate HMG–CoA. We (16) discussed the existence of a pathway as proposed by Emmanuel and Robblee (153), starting from propionate, that was incorporated into cholesterol in rat liver (154). According to these authors' hypothesis, propionyl-CoA could condense with propionaldehyde in a reaction analogous to the formation of malate from acetyl-CoA and glyoxylate, followed by the mutation of a methyl group catalyzed by a mutase in analogy to the conversion of L-glutamate to *threo*-β-methyl-L-aspartate. Oxidation of this hypothetical intermediate would yield MVA. Some conversion of propionate into 3-hydroxypropionate (and traces of acetate) in lima beans (*Phaseolus limensis*) with acrylic acid as an intermediate has been described (155). However, a vitamin B_{12}-dependent mutase activity, which would have led to the formation of a methylmalonyl derivative and finally to succinate, could not be detected. Thus, we should probably abandon those alternative models and return to the Rohmer pathway (125) as outlined above, or, alternatively, to the pathway proposed by Hano and his colleagues (135,136).

Similarly, the presence of the Rohmer pathway in plants could provide a further explanation for the observations obtained with the HMGR inhibitor mevinolin, *viz.* its great efficiency in blocking cytoplasmic sterol biosynthesis, but its low efficiency in affecting ubiquinone biosynthesis, and its practically complete inefficiency in blocking the accumulation of isoprenoids and prenyl lipids of the chloroplast, as summarized and discussed earlier (16, 23). It could also explain the puzzling observation that, in contrast to phytosterols, prenyl-substituted coumarins were not labeled from [2-^{14}C]acetate nor from [2-^{14}C]MVA in elicitor-treated cultures of *Ammi majus*, suggesting that the DMAPP needed as substrate for the (plastid?) umbelliferone: dimethylallyl transferase is formed from IPP synthesized *de novo* within the plastid, rather than from IPP imported into the plastid from the cytosol (156). Elicitor-induced inhibition of phytosterol biosynthesis should occur by specific inhibition of one of the enzymes on the cytosolic pathway from MVA to DMAPP (156). IPP was similarly accepted as an effective substrate for prenyl lipid synthesis as CO_2 when testing isolated chloroplasts from the unicellular green alga *Acetabularia* (77), whereas "conventional" IPP precursors were not. This observation can easily be explained by the existence of an alternative pathway.

MVA and IPP were more rapidly taken up by proplastid-like plastids from 70S ribosome-deficient, bleached leaves of rye seedlings grown at a nonpermissive temperature of 32°C (157), than by chloroplasts from green seedlings cultivated at 22°C. But in contrast to IPP, MVA was not incorporated into lipids by either type of isolated organelle. However *in vivo*, MVA was incorporated into prenyl lipids such as plastoquinone, phylloquinone, and lutein in leaf sections of rye seedlings (157). In bleached leaves the rate of incorporation was strikingly higher than in green leaves. If any interpretation is to be made of such discrepancies between the situation *in vitro* and *in vivo*, these data seem to indicate that divergent types of plastid differentiation might be associated with fundamental changes of the metabolite flow of isoprenoid precursors between different products and compartments and with changes of import into the plastid compartment (157). The presence of plastidic MVA kinase and MVAP kinase in addition to cytosolic activities has been claimed from latency experiments using immature chloroplasts from barley (158). However, the cloning of a cDNA from *Arabidopsis thaliana* encoding MVA by complementation of a yeast mutant (159), and corresponding Southern blot analyses have not yet provided any evidence for the existence of a further MVA kinase gene, for instance coding for a organelle-specific isozyme. The cloning of corresponding genes from evolutionarily distant plants is therefore highly desirable in order to possibly detect isogenes. (For literature on early controversies on the presence of MVA kinase isoforms in plant cell, see Ref. 14.) Single MVA kinase genes have also been cloned from rat liver (160), human tissue (161), and from yeast (162). However, recently, evidence has been presented of a predominant localization of a 42 kDa MVA kinase in rat peroxisomes, immunologically distinct from a cytosolic 40 kDa enzyme, as well as from a 46 kDa enzyme in mitochondria (163). Thus, it cannot be excluded that evolutionarily distinct isozymes of MVA kinase exist in eukaryotes.

In a recent report, Albrecht and Sandmann (164) have used maize etioplasts to study the light-stimulated biosynthesis of carotenoids. Enzyme activities downstream from MVA were determined. It appears as if exclusively IPP isomerase is affected by light (164). Unfortunately, absolute values for plastidic activities of MVA kinase, MVAP kinase, and MVAPP decarboxylase were not given (164). From their incorporation data using acetate and pyruvate as precursors, Heintze *et al.* (165), concluded that genes encoding the chloroplast MVA pathway might be expressed only at an early stage of chloroplast development (which, however, does not resolve the abovementioned dilemma of having no HMGR gene coding for a protein with a leader peptide typical of translocation into plastids). Future work might reveal that, depending on the plant species and their developmental stage, different strate-

gies might be used to ensure a sufficient IPP synthesis in the cytoplasm and in the organelles. If such anaplerotic and/or compartmentalized pathways coexist, then we have to look for some molecular switch or sensor responsible for activating genes for different biosynthetic routes.

In order to explain such contradictory observations, it might also be possible to consider substrate channeling with no relase or access of intermediates (and of inhibitors), for instance, through a close arrangement of enzymes into metabolons within an end-product specific reaction sequence, like in carbohydrate metabolism (143), e.g., at the surface of membranes. This could be envisaged to occur even in the same subcellular compartment. A synthesis of products finally localized in plastids and mitochondria outside of the organelle has been proposed (166). The Golgi apparatus has recently been discussed as such a subcellular site of synthesis for prenylquinones (166); this is somewhat different from (167), or even in sharp contrast (168) to earlier findings (see Refs. 11,14 for further literature). This would require the presence of specific transport systems in the envelope membranes of plastids and mitochondria as well. No clear evidence of their existence has been presented yet.

CONCLUSION

From the observations discussed above, which are partially in conflict with each other, it should become obvious that we are still quite a long way from completely understanding how the individual enzymes/genes of the multibranched isoprenoid pathway in plants are compartmentalized or are interacting. In some way, we have to revise well established textbook knowledge which, as far as plant biochemistry is concerned, was not always experimentally proven, but rather deduced by analogy from results obtained with animal tissue or yeast. The co-existence of alternative machineries for the synthesis of one and the same isoprenoid precursor, be it IPP or MVA, is an even more intriguing challenge for future studies in biochemistry, molecular biology and physiology. This possibly extends to evolutionary aspects (169,170), for instance, to the symbiotic origins of organelles (171,172) and to the conservation of (prokaryotic?) pathways in higher plants. Sometimes things are not as simple as they look at first sight!

ACKNOWLEDGMENTS

Previous work in the author's laboratory at the University of Karlsruhe was generously supported by the Deutsche Forschungsgemeinschaft. Ongoing studies at the Institut de Biologie Moléculaire des Plantes (Strasbourg) are made possible by the support of the C.N.R.S. The helpful discussions with Prof. Michel Rohmer (Mulhouse, France), Drs. Alain Cartayrade and Matthias Schwarz (Zürich), Dr. Robert van der Heijden (Leiden), Prof. Albert Boronat (Barcelona), Prof. Wilhelm Gruissem (Berkeley), Prof. Pierre Benveniste and Dr. Hubert Schaller (Strasbourg), and Prof. Shinichi Ueda (Kyoto) on their partially unpublished work are greatly appreciated. Special thanks are given to Prof. W. David Nes and Prof. Ed Parish for the invitation to the AOCS symposium on sterols, and to Dr. Jean-Caude Kader and Prof. Paul Mazliak for the opportunity to present a major part of this article to the participants of the 11th International Plant Lipid Meeting.

REFERENCES

1. Conolly, J.D., and Hill, R.A. (1992) *Dictionary of Terpenoids*, Chapman and Hall, New York.
2. Wahlberg, I., and Enzell, C.R. (1987) *Nat. Prod. Rep. 4*, 237–276.
3. Wolf, D.E., Hoffman, C.H., Aldrich, P.E., Skeggs, H.R., Wright, L.D., and Folkers, K. (1956) *J. Am. Chem. Soc. 78*, 4499.
4. Tavormina, P.A., and Gibbs, M.H. (1956) *J. Am. Chem. Soc. 78*, 6210.
5. Tavormina, P.A., Gibbs, M.H., and Huff, J.W. (1956) *J. Am. Chem. Soc. 78*, 4498–4499.
6. Goodwin, T.W. (ed.) (1970) *Natural Substances Formed Biologically from Mevalonic Acid*, Biochem. Soc. Symp. No. 29, Liverpool 1969, Academic Press, New York.
7. Banthorpe, D.V., Charlwood, B.V., and Francis, M.J.O. (1972) *Chem. Rev. 72*, 115–149.
8. Nes, W.R., and McKean, M.L. (1977) *Biochemistry of Steroids and Other Isopentenoids*, University Park Press, Baltimore.
9. Porter, J., and Spurgeon, S.L. (eds.) (1981) *Biosynthesis of Isoprenoid Compounds, Vol. 1*, John Wiley and Sons, New York.
10. Goad, L.J. (1983) *Biochem. Soc. Transact. 11*, 548–552.
11. Kleinig, H. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 40*, 39–59.
12. Nes, W.D., and Bach, T.J. (1985) *Proc. Roy. Soc. Lond. B 225*, 425–444.
13. Bach, T.J. (1986) *Lipids 21*, 82–88.
14. Gray, J.C. (1987) *Adv. Bot. Res. 14*, 25–91.
15. West. C. (1990) in *Plant Physiology, Biochemistry and Molecular Biology* (Dennis, D.T., and Turpin, D.H., eds.) pp. 353–369, Longman Scientific & Technical, Essex.
16. Bach, T.J., Motel, A., and Weber, T. (1990) *Rec. Adv. Phytochemistry 24*, 1–82.
17. Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schönberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3957–3961.
18. Bach, T.J., and Lichtenthaler, H.K. (1982) *Z. Naturforsch. C. 37*, 46–50.
19. Bach, T.J., and Lichtenthaler, H.K. (1983) *Z. Naturforsch. C. 38*, 212–219.
20. Nakamura, C.E., and Abeles, R.H. (1985) *Biochemistry 24*, 1364–1376.
21. Louis-Flamberg, P., Peishoff, C.E., Bryan, D.L., Leber, J., Elliot, J.D., Metcalf, B.W., and Mayer, R.J. (1990) *Biochemistry 29*, 4115–4120.
22. Bach, T.J., and Lichtenthaler, H.K. (1983) *Physiol. Plant. 59*, 50–60.
23. Bach, T.J., and Lichtenthaler, H.K. (1987) in *Ecology and Metabolism of Plant Lipids* (Fuller, G., and Nes, W.D., eds.), *Am. Chem. Soc. Symposium Series 325*, pp. 109–139, American Chemical Society, Washington, DC.
24. Hata, S., Takagishi, H., Egawa, Y., and Ota, Y. (1986) *Plant Growth Regul. 4*, 335–346.
25. Hata, S., Takagishi, H., and Kouchi, H. (1987) *Plant Cell Physiol. 28*, 709–714.
26. Maillot-Vernier, P., Gondet, L., Schaller, H., Benveniste, P., and Belliard, G. (1991) *Mol. Gen. Genet. 231*, 33–40.

27. Gondet, L., Weber, T., Maillot-Vernier, P., Benveniste, P., and Bach, T.J. (1992) *Biochem. Biophys. Res. Commun. 186*, 888–893.
28. Gondet, L. (1993) Caractérisation biochimique et cytochimique d'un mutant de tabac (*Nicotiana tabacum* L.) surproducteur de stérols. Thèse doctorale, Université Louis Pasteur, Strasbourg, France.
29. Gondet, L., Bronner, R., and Benveniste, P. (1994) *Plant. Physiol. 105*, 509–518.
30. Chappell, J., Proulx, J., Wolf, F., Cuellar, R.E., and Saunders, C. (1991) *Plant Physiol. 96 (Suppl.)*, 127.
31. Hata, S., Shirata, K., Takagishi, H., and Kouchi, H. (1987) *Plant Cell Physiol. 28*, 715–722.
32. Kyte, J., and Doolittle, R.F. (1982) *J. Mol. Biol. 157*, 105–132.
33. Narita, J.O., and Gruissem, W. (1989) *Plant Cell 1*, 181–190.
34. Caelles, C., Ferrer, A., Balcells, L., Hegardt, F.G., and Boronat, A. (1989) *Plant Mol. Biol. 13*, 627–638.
35. Learned, R.M., and Fink, G.R. (1989) *Proc. Natl. Acad. Sci. USA 86*, 2779–2783.
36. Monfar, M., Caelles, C., Balcells, L., Ferrer, A., Hegardt, F.G., and Boronat, A. (1990) *Rec. Adv. Phytochemistry 24*, 83–98.
37. Wettstein, A., Caelles, C., Boronat, A., Jenke, H.-S, and Bach, T.J. (1989) *Biol. Chem. Hoppe-Seyler 370*, 806–807.
38. Ferrer, A., Aparicio, C., Nogués, N., Wettstein, A., Bach, T.J., and Boronat, A. (1990) *FEBS Lett. 266*, 67–71.
39. Dittrich, B., Boronat, A., Ferrer, A., and Bach, T.J. (1991) *Biol. Chem. Hoppe-Seyler 372*, 528.
40. Chye, M.-L., Kush, A., Tan C.-T., and Chua, N.-H. (1991) *Plant Mol. Biol. 16*, 567–577.
41. Chye, M.-L., Tan, C.-T., and Chua, N.-H. (1992) *Plant Mol. Biol. 19*, 473–484.
42. Bach, T.J., Boronat, A., Caelles, C., Ferrer, A., Weber, T., and Wettstein, A. (1991) *Lipids 26*, 637–648.
43. Stermer, B.A., Edwards, L.A., Edington, B.V., and Dixon, R.A. (1991) *Physiol. Mol. Plant Pathol. 39*, 135–145.
44. Park, H., Denbow, C.J., and Cramer, C.L. (1992) *Plant Mol. Biol. 20*, 327–331 (1992).
45. Genschik, P., Criqui, M.-C., Parmentier, Y., Marbach, J.A., Durr, A., Fleck, J., and Jamet, E. (1992) *Plant Mol. Biol. 20*, 337–341.
46. Maldonado-Mendoza, I.E., Burnett, R.J., and Nessler, C.L. (1992) *Plant Physiol. 100*, 1613–1614.
47. Oosterhaven, K., Hartmans, K.J., and Huitzing, H.J. (1993) *J. Plant Physiol. 141*, 463–469.
48. Burnett, R.J., Maldonado-Mendoza, I.E., McKnight, T.D., and Nessler, C.L. (1993) *Plant Physiol. 103*, 41–48.
49. Bach, T.J., Wettstein, A., Boronat, A., Ferrer, A., Enjuto, M., Gruissem, W., and Narita, J.O. (1991) in *Physiology and Biochemistry of Sterols* (Patterson, G.W., and Nes, W.D., eds.), pp. 29–49, American Oil Chemists' Society, Champaign.
50. Enjuto, M., Balcells, L., Campos, N., Caelles, C., Arró, M., and Boronat, A. (1994) *Proc. Natl. Acad. Sci. USA 91*, 927–931.
51. Wong, R.J., McCormack, K., and Russell, D.W. (1982) *Arch. Biochem. Biophys. 216*, 631–638.
52. Wititsuwannakul, R. (1986) *Experientia 42*, 44–46.
53. Bach, T.J. (1987) *Plant Physiol. Biochem. 25*, 163–178.
54. Stermer, B.A., and Bostock, R.M. (1987) *Plant Physiol. 84*, 404–408.
55. Chappell, J., and Nable, R. (1989) *Plant Physiol. 85*, 469–473.
56. Chappell, J., von Lanken, C., Vögeli, U., and Bhatt, P. (1989) *Plant Cell Reports 8*, 48–52.
57. Boll, M., and Kardinal, A. (1990) *Z. Naturforsch. 45*, 973–979.
58. Wititsuwannakul, R., Wititsuwannakul, D., and Dumchong, S. (1990) *Phytochemistry 29*, 1755–1758.
59. Yang, Z., Park, H., Lacy, G.H., and Cramer, C.L. (1991) *Plant Cell 3*, 397–405.
60. Choi, D., Ward, B.L., and Bostock, R.M. (1992) *Plant Cell 4*, 1333–1344.
61. Ji, W., and Hatzios, K.K. (1992) *Pesticide Biochem. Physiol. 42*, 54–63.
62. Ji, W., Hatzios, K.K., and Cramer, C.L. (1992) *Physiol. Plant. 84*, 185–192.
63. Wilson, T.M., and Russell, D.W. (1992) *Biochem. Biophys. Res. Commun. 184*, 530–537.
64. Mackintosh, R.W., Davies, S.P., Clarke, P.R., Weekes, J, Gillespie, J.G., Gibb, B.J., and Hardie, D.G. (1992) *Eur. J. Biochem. 209*, 923–931.
65. Moore, K.B., and Oishi, K.K. (1993) *Plant Physiol. 101*, 485–491.
66. Choi, D., and Bostock, R.M. (1994) *Plant Physiol. 104*, 1237–1244.
67. Gershenzon, J., and Croteau, R. (1990) *Rec. Adv. Phytochem. 24*, 99–160.
68. Stermer, B.A., Bianchini, G.M., and Korth, K.L. (1994) *J. Lipid Res. 35*, 1133–1140.
69. Darney, B.G., Wang, Y.L., and Rodwell, V.W. (1992) *J. Biol. Chem. 267*, 15064–15070.
70. Olender, E.H., and Simoni, R.D. (1992) *J. Biol. Chem. 267*, 4223–4235.
71. Roitelman, J., Olender, E.H., Barnum, S., Dunn, W.A., and Simoni, R.D. (1992) *J. Cell Biol.* 117, 959–974.
72. Vollack, K.-U., Dittrich, B., Ferrer, A., Boronat, A., and Bach, T.J. (1994) *J. Plant Physiol. 143*, 479–487.
73. Arebalo, R.E., and Mitchell, Jr., E.D. (1984) *Phytochemistry 23*, 13–18.
74. Ramachandra Reddy, A., and Das, V.S.R. (1986) *Phytochemistry 25*, 2471–2474.
75. Ramachandra Reddy, A., and Das, V.S.R. (1987) *New Phytol. 106*, 457–464.
76. Schulze-Siebert, D., and Schultz, G. (1987) *Plant Physiol. Biochem. 25*, 145–153.
77. Bäuerle, R., Lütke-Brinkhaus, F., Ortmann, B., Berger, S., and Kleinig, H. (1990) Planta *181*, 229–233.
78. Heintze, A., Görlach, J., Leuschner, C., Hoppe, P., Hagelstein, P., Schulze-Siebert, D., and Schultz, G. (1990) *Plant Physiol. 93*, 1121–1127.
79. Soll, J., and Waegemann, K. (1993) Abstract No. 5.4.3–2, *XV International Botanical Congress, Yokohama, Japan, August 28–September 3, 1993*, Abstract Volume p. 132.
80. Boronat, A., Balcells, L., Campos, N., Enjuto, M., Lumbreras, V., Marín, C., Arró, M., Becerra, B., and Ferrer, A. (1994) Abstract L2, *2nd Symposium of the European Network on Plant Terpenoids*, Strasbourg/Bischenberg, January 23–27, 1994.
81. Jenkins, S., Callan, K., Loraine, A., Schmidt, D., and Gruissem, W. (1994) Abstract L3, *2nd Symposium of the European Network on Plant Terpenoids*, Strasbourg/Bischenberg, January 23–27, 1994.
82. Weber, T., and Bach, T.J. (1994) *Biochim. Biophys. Acta 1211*, 85–96.
83. Bach, T.J., Raudot, V., Vollack, K.-U., Weber, T., and Zeiler, S. (1994) *Plant Physiol. Biochem. 32*, 775–783.
84. Vollack, K.-U. (1994) Isolierung und Charakterisierung einer cDNA für die Acetoacetyl-CoA-Thiolase [EC 2.3.1.9] aus *Raphanus sativus* L. durch Komplementierung einer *Saccharomyces cerevisiae*-Mutante. Dissertation, Fakultät für Bio- und Geowissenschaften, Universität Karlsruhe, Germany.
85. van der Heijden, R., de Boer-Hlupá, V., Verpoorte, R., and Duine, J.A. (1994) *Plant Cell Tissue Organ Cult. 38*, 345–349.
86. van der Heijden, R., Verpoorte, R., and Duine, J.A. (1994) *Plant Physiol. Biochem. 32*, 807–812.
87. McCaskill, D., and Croteau, R. (1993) *Anal. Biochem. 215*, 142–149.

88. Suga, T., Tange, K., Iccho, K., and Hirata, T. (1980) *Phytochemistry 19*, 67–70.
89. Tange, K. (1981) *Bull. Chem. Soc. Jpn. 54*, 2763–2769.
90. Anastasis, P., Freer, I., Picken, D., Overton, K., Sadler, I., and Singh, S.B. (1983) *J. Chem. Soc. Chem. Commun.*, 1189–1191.
91. Anastasis, P., Freer, I., Overton, K., Rycroft, D., and Singh, S.B. (1985) *J. Chem. Soc. Chem. Commun.*, 148–149.
92. Banerjii, A., and Chintalwar, G.J. (1989) *Ind. J. Biochem. Biophys. 26*, 394–396.
93. Weber, T., and Bach, T.J. (1993) *Z. Naturforsch. C. 48*, 444–450.
94. Wurtele, E.S., and Nicolau, B.J. (1990) *Arch. Biochem. Biophys. 278*, 179–186.
95. Wurtele, E.S., and Nicolau, B.J. (1992) *Plant Physiol. 99*, 1699–1703.
96. Baldet, P., Alban, C., Axiotis, S., and Douce, R. (1992) *Plant Physiol. 99*, 450–455.
97. Alban, C., Baldet, P., Axiotis, S., and Douce, R. (1993) *Plant Physiol. 102*, 957–965.
98. Clauss, M., Motel, A., and Lichtenthaler, H.K. (1993) *J. Plant Physiol. 141*, 508–511.
99. Gerbling, H. (1993) *Bot. Acta 106*, 380–387.
100. Loreto, F., and Sharkey, T.D. (1993) *Planta 189*, 420–424.
101. Fall, R., and Monson, R.K. (1992) *Plant Physiol. 100*, 987–992.
102. Loreto, F., and Sharkey, T.D. (1993) *Plant Cell Environm. 16*, 563–570.
103. Silver, G.M., and Fall, R. (1991) *Plant Physiol. 96*, 563–570.
104. Sanadze, G.A., Dzhaini, G.I. and Tevzadze, T.M. (1972) *Sov. Plant Physiol. 19*, 17–20.
105. Rasmussen, R.A., and Jones, C.A. (1973) *Phytochemistry 12*, 15–19.
106. Mgaloblishvili, M.P., Khetsuriani, N.D., Kalandadze, A.N., and Sanadze, G.A. (1979) *Sov. Plant Physiol. 25*, 837–842.
107. Tingey, D.T., Evans, R., and Gumpertz, M. (1981) *Planta 152*, 565–570.
108. Monson, R.K., Hills, A.J, Zimmermann, P.R., and Fall, R. (1991) *Plant Cell Environm. 14*, 517–523.
109. Stone, B.G., Besse, T.J., Duane, W.C., Evans, C.D., and DeMaster, E.G. (1993) *Lipids 28*, 705–708.
110. Reardon, J.E., and Abeles, R.H. (1986) *Biochemistry 25*, 5609–5616.
111. Muehlbacher, M., and Poulter, C.D. (1988) *Biochemistry 27*, 7315–7328.
112. Deneris, E.S., Stein, R.A., and Mead, J.F. (1985) *J. Biol. Chem. 260*, 1382–1385.
113. Takahashi, I. and Ogura, K. (1981) *J. Biochem. (Tokyo) 89*, 1581–1587.
114. Fujii, H., Koyama, T., and Ogura, K. (1982) *J. Biol. Chem. 257*, 14610–14612.
115. Fujii, H., Koyama, T., and Ogura, K. (1983) *FEBS Lett. 161*, 257–260.
116. Takatsuji, H., Nishino, T., Miki, I., and Katsuki, H. (1983) *Biochem. Biophys. Res. Commun. 110*, 187–193.
117. Fujisaki, S., Nishino, T. and Katsuki, H. (1986) *J. Biochem. (Tokyo) 99*, 1137–1146.
118. Fujisaki, S., Nishino, T., and Katsuki, H. (1986) *J. Biochem. (Tokyo) 99*, 1327–1337.
119. Seckler, B., and Poralla, K. (1986) *Biochim. Biophys. Acta 881*, 356–363.
120. Siebert, M., Bechtold, A., Melzer, M., May, U., Berger, U., Schröder, G., Schröder, J., Severin, K., and Heide, L. (1992) *FEBS Lett. 307*, 347–350.
121. Melzer, M., and Heide, L. (1994) *Biochim. Biophys. Acta 1212*, 93–102.
122. Taylor, R.F. (1984) *Microbiol. Rev. 48*, 181–198.
123. Zhou, D., and White, R.H. (1991) *Biochem. J. 273*, 627–634.
124. Rohmer, M., Bisseret, P., and Sutter, B. (1992) *Progr. Drug Res. 37*, 271–285.
125. Rohmer, M., Knani, M., Simonin, P., Sutter, B., and Sahm, H. (1993) *Biochem. J. 295*, 517–524.
126. Ourisson, G., and Rohmer, M. (1992) in *Rheinisch-Westfälische Akademie der Wissenschaften, Vorträge Nr. 395*, pp. 21–34, Westdeutscher Verlag, Aachen.
127. Horbach, S., Sahm, H., and Welle, R. (1993) *FEMS Microbiol. Lett. 111*, 135–140 (1993).
128. Flesch, G., and Rohmer, M. (1988) *Eur. J. Biochem. 175*, 405–411.
129. Rohmer, M., Sutter, B., and Sahm, H. (1989) *J. Chem. Soc. Chem. Commun.*, 1471–1472.
130. Rohmer, M., Bisseret, M.P., and Sutter, B. (1991) *Progr. Drug Res. 37*, 271–285.
131. Rohmer, M. (1993) *Pure Appl. Chem. 65*, 1293–1298.
132. Simonin P., Jürgens, U.W., and Rohmer, M. (1992) *Tetrahedron Lett. 33*, 3629–3632.
133. Stampf, P., Herrmann, D., Bisseret, P., and Rohmer, M. (1990) *Tetrahedron 47*, 7081–7090.
134. Peiseler, B., and Rohmer, M. (1991) *J. Chem. Soc. Perkin Trans. I*, 2449–2453.
135. Hano, Y., Ayukawa, A., Nomura, T., and Ueda, S. (1994) *Naturwissenschaften 81*, 260–262.
136. Hano, Y., Ayukawa, A., Nomura, T., and Ueda, S. (1994) *J. Am. Chem. Soc. 116*, 4189–4193.
137. Cartayrade, A., Schwarz, M., Jaun, B., and Arigoni, D. (1994) Abstract P1, *2nd Symposium of the European Network on Plant Terpenoids,* Strasbourg/Bischenberg, January 23–27, 1994.
138. Flesch, V., Jacques, M., Cosson, L., Teng, B.P., Petiard, V., and Balz, J.P. (1992) *Phytochemistry 31*, 1941–1945.
139. Schwarz, M. (1995) Dissertation Nr. 10951, Eidgenössische Technische Hochschule, pp. 84–171, Zürich, Switzerland.
140. Soler, E., Clastre, M., Bantignies, B., Marigo, G., and Ambid, C. (1993) *Planta 191*, 324–329.
141. Stivers, J.T., and Washabaugh, M.W. (1993) *Biochemistry 32*, 13472–13482.
142. Lee, T.C., and Langston-Unkefer, P.J. (1985) *Plant Physiol. 79*, 242–247.
143. Miernyk, J.A. (1990) in *Plant Physiology, Biochemistry and Molecular Biology* (Dennis, D.T., and Turpin, D.H., eds.) pp. 77–100, Longman Scientific & Technical, Essex.
144. Kelley, P.M. (1989) *Plant Mol. Biol. 13*, 213–222.
145. Peschke, V.M., and Sachs, M.M. (1993) *Mol. Gen. Genet. 240*, 206–212.
146. Randall, D.D., and Miernyk, J.A. (1990) in *Methods in Plant Biochemistry Vol. 3, Enzymes of Primary Metabolism* (Lea, P.J., ed.) pp. 175–192, Academic Press, London.
147. Hoppe, P., Heintze, A., Riedel, A., Creutzer, C., and Schultz, G. (1993) *Planta 190*, 253–262.
148. McHale, N.A., Hanson, K.R., and Zelitch, I. (1988) *Plant Physiol. 88*, 930–935.
149. Tse, J.M.-T., and Schloss, J.V. (1993) *Biochemistry 32*, 10398–10403.
150. Gutkowski, J.A., and Lienhard, G.E. (1976) *J. Biol. Chem. 251*, 671–684.
151. Kleinig, H. (1987) in *The Metabolism, Structure, and Function of Plant Lipids* (Stumpf, P.K., Mudd, J.B., and Nes, W.D., eds.) pp. 37–43, Plenum Press, New York.
152. Lütke-Brinkhaus, F., and Kleinig, H. (1987) *Planta 171*, 406–411.
153. Emmanuel, B., and Robblee, A.R. (1984) *Int. J. Biochem. 16*, 907–911.
154. Davis, A.R. (1978) *Steroids 31*, 593–600.
155. Halarnkar, P.P., Wakayama, E.J., and Blomquist, G.J. (1988) *Phytochemistry 27*, 997–999.

156. Fulton, D.C., Kroon, P.A., Matern, U., Threlfall, D.R., and Whitehead, I.M. (1993) *Phytochemistry 34,* 139–145.
157. Gölz, P., and Feierabend, J. (1993) *Z. Naturforsch. C. 48,* 886–895.
158. Preiss, M., and Schultz, G. (1994) *Plant Physiol. 105 (Suppl.),* 65.
159. Riou, C. (1994) Isolement et caractérisation de l'ADNc codant pour la mévalonate kinase d'*Arabdopsis thaliana* par complémentation chez la levure *Saccharomyces cerevisiae.* Thèse doctorale, Université de Poitiers, France.
160. Tanaka, R.D., Lee, L.-Y., Schafer, B.L., Kratunis, V.J., Mohler, W., Robinson, G.W., and Mosley, S.T. (1990) *Proc. Natl. Acad. Sci. USA 87,* 2872–2876.
161. Schafer, B.L., Bishop, R.W., Kratunis, V.J., Kalinowski, S.S., Mosley, S.T., Gibson, K.M., and Tanaka, R.D. (1992) *J. Biol. Chem. 267,* 13229–13238.
162. Oulmouden, A., and Karst, F. (1991) *Curr. Genet. 19,* 9–14.
163. Biardi, L., Sreedhar, A., Zokaei, A., Vartak, N.B., Bozeat, R.L., Shakelford, J.E., Keller, G.-A., and Krisans, S.K. (1994) *J. Biol. Chem. 269,* 1197–1205.
164. Albrecht, M., and Sandmann, G. (1994) *Plant Physiol. 105,* 529–534.
165. Heintze, A. Riedel, A., Aydogdu, S., and Schultz, G. (1994) *Plant Physiol. Biochem. 32,* 791–797.
166. Swiezewska, E., Dallner, G., Andersson, B., and Ernster, L. (1993) *J. Biol. Chem. 268,* 1494–1499.
167. Janiszowska, W. (1987) *Phytochemistry 26,* 1403–1407.
168. Soll, J., Schultz, G., Joyard, J., Douce, R., and Block, M.A. (1985) *Arch. Biochem. Biophys. 238,* 290–299.
169. Nes, W.R., and Nes, W.D. (1980) *Lipids in Evolution,* Plenum Press, New York.
170. Nes, W.D., and Venkatramesh, M. (1994) in *Isopentenoids and Other Natural Products: Evolution and Function* (Nes, W.D., ed.), ACS Symposium Series, 562, 85–99, American Chemical Society, Washington DC.
171. Morden, C.W., Delwiche, C.F., Kuhsel, M., and Palmer, J.D. (1992) *BioSystems 28,* 75–90.
172. Cavalier-Smith, T. (1992) *BioSystems 28,* 91–106.

[Received August 24, 1994, and in revised form January 16, 1995; Revision accepted January 17, 1995]

Developmental Regulation of Sterol Biosynthesis in *Zea mays*[1,2]

De-an Guo[3], M. Venkatramesh and W. David Nes*

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409

ABSTRACT: Sixty-one sterols and pentacyclic triterpenes have been isolated and characterized by chromatographic and spectral methods from *Zea mays* (corn). Several plant parts were examined; seed, pollen, cultured hypocotyl cells, roots, coleoptiles (sheaths), and blades. By studying reaction pathways and mechanisms on plants fed radiotracers ([2-^{14}C]mevalonic acid, [2-^{14}C]acetate, and [2-^{3}H]acetate), and stable isotopes (D_2O), we discovered that hydroxymethylglutaryl CoA reductase is not "the" rate-limiting enzyme of sitosterol production. Additionally, we observed an ontogenetic shift and kinetic isotope effect in sterol biosynthesis that was associated with the C-24 alkylation of the sterol side chain. Blades synthesized mainly 24α-ethyl-sterols, sheaths synthesized mainly 24-methyl-sterols, pollen possessed an interrupted sterol pathway, accumulating 24(28)-methylene-sterols, and germinating seeds were found to lack an active *de novo* pathway. Shoots, normally synthesizing (*Z*)-24(28)-ethylidine-cholesterol, after incubation with deuterated water, synthesized the rearranged double-bond isomer, stigmasta-5,23-dien-3β-ol. Examination of the mass spectrum and ^{1}H nuclear magnetic resonance spectrum of the deuterated 24-ethyl-sterol indicated the Bloch-Cornforth route originating with acetyl-CoA and passing through mevalonic acid to sterol was not operative at this stage of development. An alternate pathway giving rise to sterols is proposed.

Lipids 30, 203–219 (1995).

The sterol compositions of *Zea mays* anatomy have been studied extensively (1–23). Sitosterol, stigmasterol, campesterol, and 24-epicampesterol were the major sterols isolated from the plant (8–10,20). Phytosterol production was found to involve irreversible carbon flux from acetyl-CoA to hydroxymethylglutaryl CoA (HMG–CoA) to mevalonic acid to isopentenoids (Bloch-Cornforth pathway) and then into a criss-crossing grid of sterol transforming enzymes of low sterol specificity until equilibrium (sterol homeostasis) was established (24,25). Sitosterol production was shown to be controlled by cycloartenol availability (Fig. 1), which presumably was controlled by the enzyme regulating conversion of HMG-CoA to mevalonic acid (HMG–CoA reductase) (24–26). The stereochemistry of C-24 alkylation was studied and found to involve multiple reaction pathways (Fig 2): 24β-methyl-sterols were formed by either the Δ^{23}- or Δ^{25}-transmethylation route whereas 24α-methyl-sterols were formed by the $\Delta^{24(28)}$-transmethylation route (18,20). Thus far, the Δ^{23}-transmethylation pathway has been demonstrated only in corn.

In our previous studies on phytosterol distribution and biochemistry (27–32), we recognized a phenomenon different from that reported for corn. That is, phytosterol genesis may be controlled during plant development at two levels of carbon flux: (i) cycloartenol production, which may be influenced either by mevalonic acid availability or carbon flux *via* the "Popják shunt" (27). This novel shunt, which we demonstrated for the first time in plants, allows for amino acids (33) and sugars (34) to be introduced into isopentyl pyrophosphate by a route that by-passes mevalonic acid as an intermediate; and (ii) cycloartenol transformation to 24-alkyl-sterols (sitosterol). This latter pathway may be controlled by the turnover rate and function of the Δ^5-sterol end products and the stoichiometry of sterol and cofactor (oxygen, *S*-adenosyl-L-methionine; and NADPH) associated with enzymes that act selectively on the sterol substrate (35,36). Both aspects of control regulate the activity of enzymes and the ordering of sterol intermediates that give rise to taxa-specific pathways from cycloartenol to 24-alkyl-sterols (35). That corn should be different from other plants in its sterol synthetic capabilities was puzzling, which led us to reinvestigate the composition and biosynthesis of sterols from corn in more detail.

In the present study, we examined the sterol compositions of several anatomical parts of *Zea mays*. Using high-performance liquid chromatography (HPLC) methods not available to earlier investigators studying corn, we identified most of the sterols reported by them and several new ones. One of the new sterols, 24β-ethyl-clerosterol, is generally thought to be synthesized only by primitive plants (37). We confirmed that changes occurred in sterol content during germination and seedling development (8–12). We also studied sterol biosynthesis using a variety of radiotracers and stable isotopes. Our

[1]Dedicated to the distinguished insect steroid biochemist Dr. James A. Svoboda on occasion of his sixtieth birthday.

[2]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

[3]Visiting Professor. Permanent address: Department of Pharmacognosy, Beijing Medical University, Beijing 100083, China.

* To whom correspondence should be addressed at Department of Chemistry and Biochemistry, Texas Tech University, P.O. Box 4260, Lubbock, TX.

Abbreviations: DBED, *N,N'*-dibenzylethylenediammonium; GLC, gas–liquid chromatography; HMG–CoA, hydroxymethylglutaryl CoA; HPLC, high-performance liquid chromatography; KIE, kinetic isotope effect; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; RRT_c, relative retention time, relative to cholesterol; SAM, *S*-adenosyl-L-methionine; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

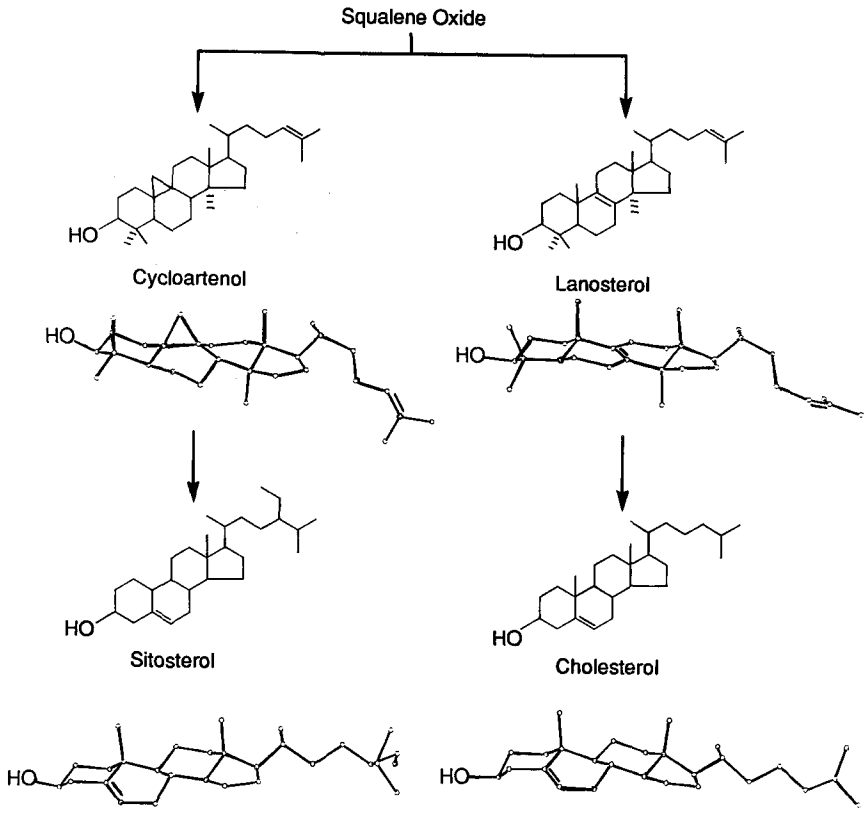


FIG. 1. Two-pathway model of sterol transformation. Both the conventional and conformational view (X-ray crystallographic structures are from Griffin and Nes, unpublished) of the sterol molecules are shown.

results indicate that synthesis of 24-ethyl-sterols is kinetically favored over 24-methyl-sterols at some, but not at all, stages of corn development; seeds do not synthesize sterols *de novo*, but they provide as much as 70% of the shoot sterol for early seedling growth. A strong kinetic isotope effect (KIE) was observed in the C-24 alkylation pathway of early maturing shoots after incubation of germinating seeds with deuterated water. This result indicates that structurally related 24-alkyl-sterol isomers are, in fact, generated by a single C-24 methyl-transferase and that C-24 alkylation may be a key regulatory step in phytosterol synthesis. Pulse labeling experiments using eight-day blades and sheaths confirmed the regulatory importance of C-24 alkylation pathways in sterol transformation. These experiments also demonstrated that the $\Delta^{24(28)}$-methylene-sterol pathway is kinetically favored in blades whereas the $\Delta^{23(24)}$-24-methyl-sterol pathway is kinetically favored in sheaths. The deuterated water incubations also indicated the Bloch-Cornforth pathway (37) involving the conversion of acetyl-CoA to HMG–CoA to sterols was down-regulated in early maturing shoots and some alternate pathway operated in the shoot to enable carbon flux into sterol.

The trivial naming and systematic nomenclature for sterols continue to be an area of much debate (reviewed in Ref. 36). Two sets of rules are now in use; one that is based on a combination of historical, chemical, and biosynthetic reasoning, the Nes system (Fig. 3) (36,37); the other follows an archaic numbering system that establishes priorities based on revised IUPAC recommendations (38).

The structural features characteristic of phytosterols include supernumerary alkyl groups at C-24 with both α and β chirality. In the Nes system (36,37), the cholestane skeleton (as the hydrocarbon) is used as the building block onto which substituents are added or are removed. The α/β-nomenclature of the nucleus is different from the one used in the side chain (36). The A/B-ring junction is assumed to be *trans* oriented (5α-H and 10β-CH_3). The α/β-system and the hydroxyl group at C-3 is β-oriented (equatorial) and is preferred to the R/S-system for distinguishing relative stereochemistry. Thus sitosterol [not β-sitosterol, β is dropped in common usage (37)] is 24α-ethyl-cholest-5-en-3β-ol [alternatively 24(*R*)-24-ethyl-cholest-5-en-3β-ol] or simply 24α-ethyl-cholesterol. Two other sterol skeletons may be used as necessary, ergostane (24-methyl-cholestane) and stigmastane (24-ethyl-cholestane). Neither the configuration at C-24 nor the presence of the Δ^{22}-bond is implied when using the ergostane or stigmastane nomenclature, even though ergosterol has a Δ^{22}-bond and 24β-methyl group and stigmasterol has a Δ^{22}-bond and 24α-ethyl group. The 4,4-dimethyl-sterol intermediates, such as cycloartenol (trivial name), have been viewed as triterpenoids by IUPAC recommendations and natural prod-

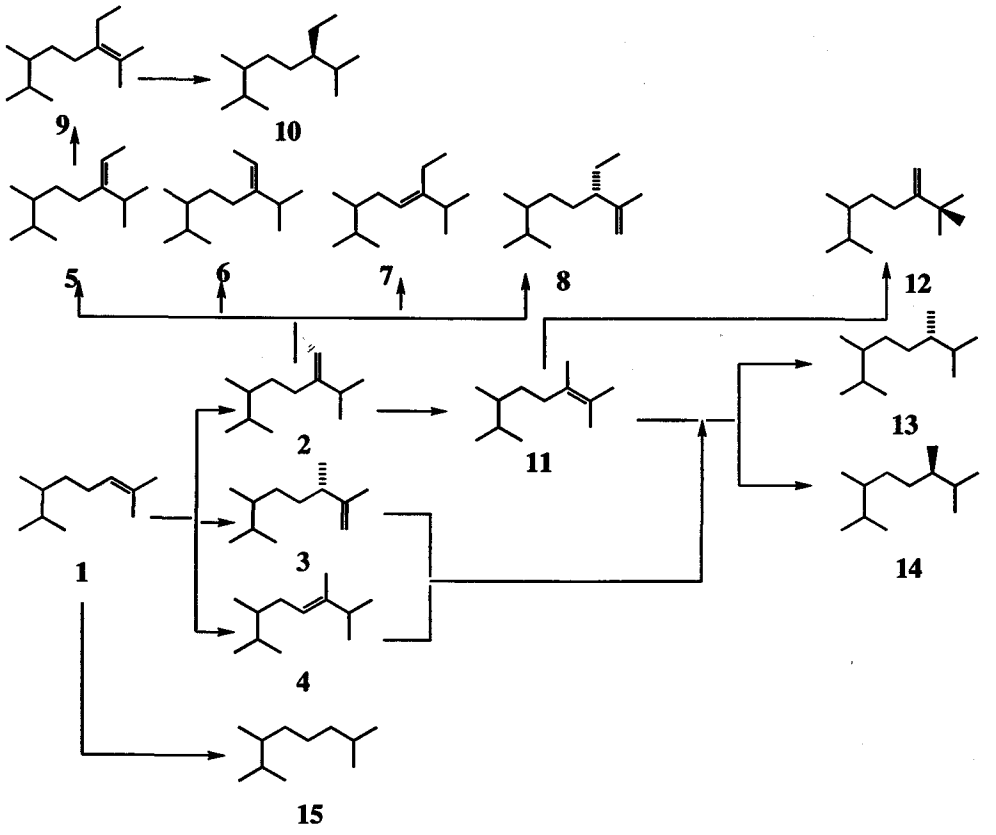


FIG. 2. Pathways of C-24 alkylation and reduction in sterol side-chain transformations; alkylation pathway involving structures **1** and **2** is operationally defined as the $\Delta^{24(28)}$-methylene pathway; alkylation pathway involving structures **1** and **3** is operationally defined as the $\Delta^{23(24)}$-pathway; alkylation pathway involving structures **1** and **4** is operationally defined as the $\Delta^{25(27)}$-pathway.

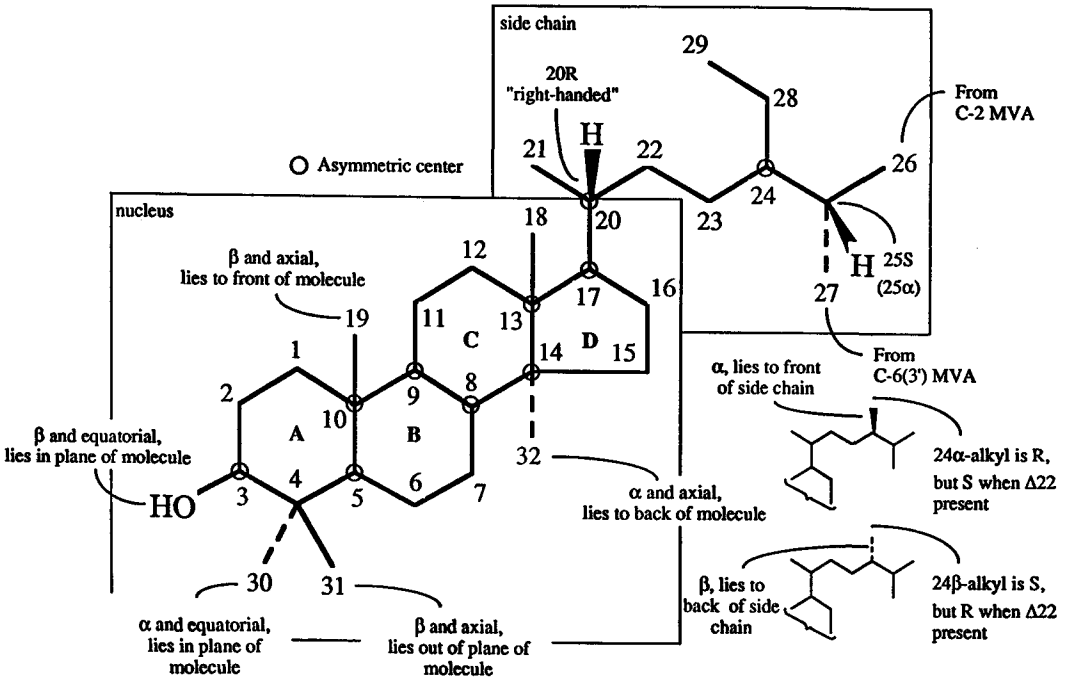


FIG. 3. Nes system (modified from Fieser and Fieser, *cf.* Ref. 37) for the numbering of carbon atoms in the sterol constitution. MVA, mevalonic acid.

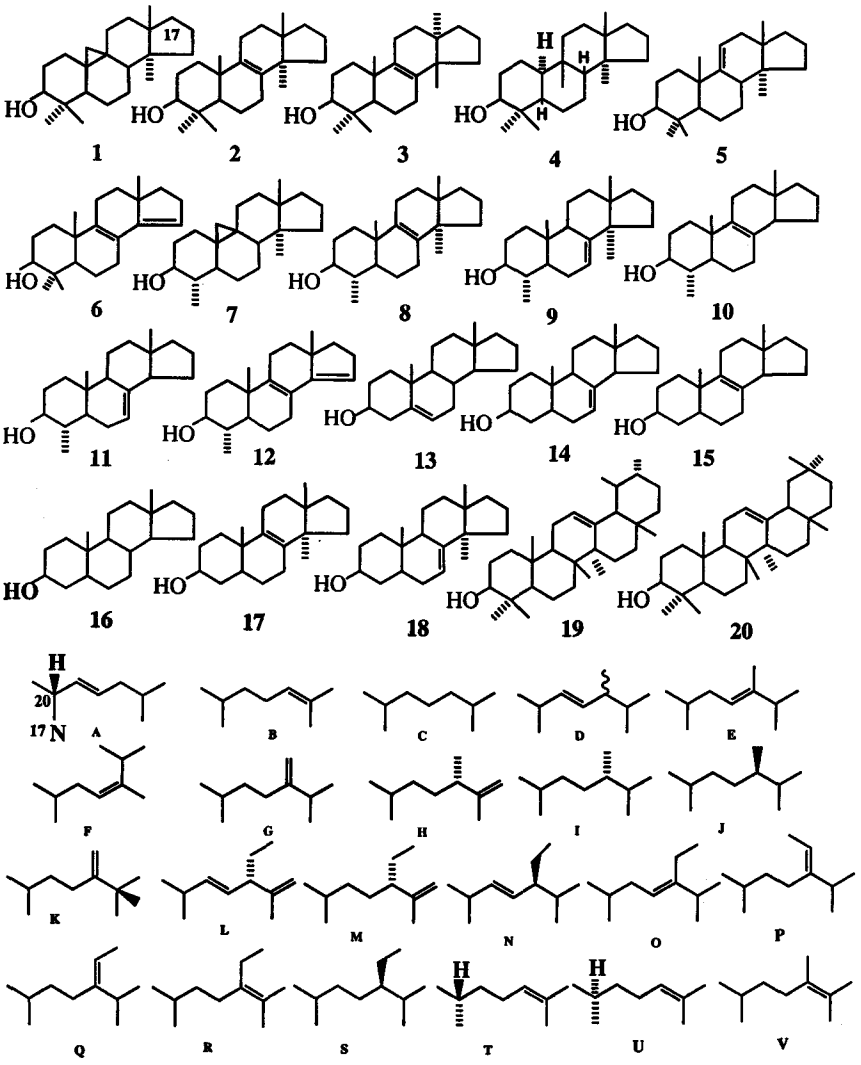


FIG. 4. Structures of sterols and triterpenoids discussed in text.

ucts chemists. We view cycloartenol as a sterol on biosynthetic grounds and refer to it formally as 4,4,14-trimethyl-9β,19- cyclo-5α-cholest-24-en-3β-ol. Alternatively, euphol is a triterpenoid, even though chemically it may be considered a structural isomer of cycloartenol. In order to save space, sterol structures are shown in Figure 4 by a number (nucleus) and letter (side chain).

MATERIALS AND METHODS

Zea mays (Lot No. 7054; Asgrow Seed Co., Stonington, IL) seeds were surface sterilized by soaking in 10% Chlorox bleach for 30 min. Seeds were then soaked in running tap water for 16 h. Seeds were germinated in trays of moistened paper towels overlaid with a second tray. The seedlings were grown in the dark for up to eight days. Seedlings with shoots and roots of similar lengths (±0.5 mm) were harvested from each tray. Three-day shoots were excised at their base. Four-day shoots were cut at the first node, and the top parts were then dissected into sheaths (coleoptiles) and blades. Four-day roots were cut 3–4 mm from the tip and at the base of the root connecting to the seed, producing two parts: the root cap and the mature root system. Eight-day shoots were dissected in a similar manner as four-day shoots. Eight-day roots were dissected at the base of the root. Friable corn cells used for tissue culture were derived from seedling hypocotyl tissue and grown on M&S medium supplemented with 3% sucrose, 2 mg/L 2,4-D, and 7 g/L agar to produce calli, and were transferred once a month onto fresh agar plates. Calli were used as inoculum for cells cultured in 1L-Erlenmeyer flasks on a shaker platform at 160 rpm, as described (39).

Incubations with labeled substrates were performed as follows. Typically fifty eight-day seedlings were grown in the dark. The blades and sheaths were separated from the seed and each group incubated in Petri dishes containing either 7 mL water or 5 mL phosphate buffer (0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 adjusted to pH 7.4). The blades and sheaths were placed in a gentle draft to enhance transpiration and up-

take of the solution during the incubation period. Additional water was supplied to the Petri dish as necessary. We observed that the addition of phosphate buffer promoted uptake of mevalonic acid into the plants. DL-[2-^{14}C]Mevalonic acid as the *N,N'*-dibenzylethylenediammonium salt (2–10 mCi/mmol) and [2-^{14}C]acetic acid as the sodium salt (40–60 mCi/mmol) were purchased from Amersham (Chicago, IL). [^{3}H]Acetic acid as the sodium salt (2–5 Ci/mmol) was purchased from NEN (Boston, MA), and D_2O (99.9% D) was purchased from Cambridge Isotopes (Andover, MA).

Sterols were isolated and analyzed in the same manner as described previously (40–42). In our experiments, we now routinely saponify the plant material directly in methanolic KOH (rather than extract the plant material in organic solvent e.g., with acetone prior to saponification). We compared the protocols reported in the literature (24) for isolating sterols from corn with our method and found that there was no quantitative difference in the amount of sterol released from the plant; also, we found that saponification of cultured cells and yeast was necessary to release most of the sterol from the cells (39,43).

The instrumentation and techniques for HPLC and gas–liquid chromatography (GLC) have been described earlier (41,44,45). GLC/mass spectrometry (MS) was performed by Dr. Robert A. Norton (USDA, Peoria, IL) on a Hewlett-Packard 5890 series II gas chromatograph (San Fernando, CA) with 5970 MSD at 70 ev. The capillary column for GLC was a 15-m DB5MS (J&W Scientific, Folsom, CA) column. The temperature program was as follows: 170°C for 1 min ramping to 270°C at 20°C/min, isothermal at 270°C for 3 min, ramping to 280°C at 2°C/ min, then isothermal at 280°C for 6 min. The scan range was 50–550, the injector port was kept at 250°C, and the transfer line at 280°C. ^{1}H nuclear magnetic resonance (NMR) spectroscopy was performed on samples dissolved in $CDCl_3$ at ambient temperature on either a Bruker AF-300 or Bruker AF-200 NMR spectrometer (Billerica, MA) with tetramethylsilane as internal standard.

Key to our developmental and kinetic studies on phytosterol biosynthesis was our ability to isolate sterols from corn plants in pure form. To that end, we developed a series of chromatographic steps using thin-layer chromatography (TLC) and reversed-phase HPLC methods (41,44,45). TLC will separate compounds based on the degree of substitution at C-4. TLC was used first to produce three classes of sterols: 4,4-dimethyl-, 4-monomethyl-, and 4-desmethyl-sterols (41). HPLC was used next to separate sterols based on the size and stereochemistry of the molecule (41,45). GLC may be used to provide preliminary characterization of the sterol and of its purity (41,45). Table 1 shows the various constitutional, geometric and diastereoisomeric features of the sterol molecule that may be distinguished by using a combination of chromatographic systems. The instrumental conditions used in Table 1 are as follows: reverse-phase HPLC; A—operated on a 25 cm Whatman partisphere C-18 column (5 mm par-

TABLE 1
Chromatographic and Spectral Properties of Sterol Isomers[a]

Sterol	Structure	HPLC (α_c)			GC (RRT_c)	
		A	B	C	A	B
Cycloartenol	1A	1.12	1.10	1.13	1.82	2.08
Lanosterol	2A	1.04	1.00	0.99	1.65	1.82
Euphol	3U	0.95	ND	ND	1.46	ND
Tirucallol	3T	0.99	ND	ND	1.57	ND
Parkeol	5A	1.04	1.00	0.99	1.79	ND
Cucurbitacin	4A	0.98	ND	ND	1.50	ND
24-Methylene-cycloartanol	1G	1.19	ND	1.12	2.10	2.29
Cyclolaudenol	1H	1.15	ND	1.04	2.10	2.29
Cyclosadol	1E	1.19	ND	1.17	2.10	2.29
Cyclobranol	1V	1.29	ND	1.25	2.49	2.82
24-Ethyl-desmosterol	13R	1.05	ND	ND	1.74	ND
Fucosterol	13P	1.01	0.90	1.00	1.60	1.76
24-Ethyl-cholesta-5,(*E*)23(24)-dienol	13O	1.05	ND	ND	1.52	ND
Isofucosterol	13Q	1.06	0.93	1.00	1.64	1.80
Clerosterol	13M	0.99	ND	1.00	1.54	ND
Campesterol	13J	1.11	0.99	1.16	1.29	ND
24-Epicampesterol	13I	1.11	1.04	1.16	1.29	ND
24-Methyl-cholesta-5,(*Z*)23(24)-dienol	13F	0.87	0.70	0.92	1.26	1.40
24-Methyl-cholesta-5,(*E*)23(24)-dienol	13E	0.87	0.83	0.95	1.26	1.40
Brassicasterol	13D(24β)	ND	0.88	0.91	1.12	ND
24-Epibrassicasterol	13D(24α)	ND	0.80	0.86	1.12	ND
α-Amyrin	19	ND	ND	1.28	1.82	ND
β-Amyrin	20	ND	ND	1.13	1.62	ND

[a]For instrumental conditions, see Materials and Methods section; ND, not determined; for structures, see Figure 4. HPLC, high-performance liquid chromatography; GC (RRT_c), gas chromatography (relative retention time, relative to cholesterol).

ticle size, 4.6 mm i.d.) eluted with MeOH/H_2O (96:4, vol/vol) at 40°C at a flow rate of 1.5 mL/min; B—operated on a 25 cm TSK gel C-18 column (Toya Soda Manufacturing Co., Tokyo, Japan; 5 mm particle size, 4.6 mm i.d.) eluted with MeOH/*i*-PrOH (4:1, vol/vol) at room temperature at a flow rate of 1.00 mL/min; C—operated on a 25 cm Zorbax C-18 column (Dupont, Boston, MA; 5 mm particle size, 4.6 mm i.d.) eluted with 100% MeOH at room temperature at a flow rate of 1.00 mL/min. GLC; A—performed on a three-foot spiral 3% SE-30 packed column operated isothermally at 245°C; B—performed on a capillary 15 M HP-50 column operated isothermally. Cycloartenol may be separated from β-amyrin by use of a Whatman C-18 column operated at low temperature (10°C) at a flow rate of 1.00 mL/min eluted with MeOH/H_2O (96:4, vol/vol).

RESULTS AND DISCUSSION

Sterol composition. The first studies were designed to establish the sterol composition of corn. We germinated several batches of seeds for four to nine days and pooled the plants. The seedlings were anatomically dissected into sheaths, blades, and roots. From this material, trace compounds could be isolated and characterized. As shown in Table 2, we identified approximately sixty sterols. The identity of each sterol was confirmed by matching their GLC behavior and mass spectra with data obtained on authentic specimens. There were several sterols occurring at trace levels that we were unable to identify. Table 2 showed that we isolated many of the same sterols identified earlier by others (1–23). Several sterols isolated by us were new corn sterols: for instance, 24(28)-methylene-25-methyl-cholesterol, cholesta-5,22-dienol, and 22-dehydroclerosterol (stigmast-5,22,25-trienol). Ergost-23-enol was a new naturally occurring sterol (Table 3). The ^1H NMR data of selected sterols isolated from corn are given in Table 3. The assignments of the ^1H NMR signals for C-26, C-27, C-28, and C-29, reported in Table 3, are based on recent labeling experiments with stable isotopes (39,46,47). The ^{13}C NMR spectrum in Figure 5 shows that the configuration of the C-24 alkyl group may be distinguished by the chemical shift of C-29. The 24-methyl-cholesterol samples were shown to be epimeric mixtures by HPLC and ^1H NMR (Fig. 6). The ratio of 24α- to 24β-methyl cholesterol varied depending on the stage of seedling development. We could not detect cyclolaudenol (limit of detection, 0.001% of the total sterol) in any plant part. However, we detected codisterol and two doubly bioalkylated $\Delta^{25(27)}$-24-ethyl-sterols. The β-configuration of the 24-ethyl group was confirmed by ^1H NMR (Table 3). We isolated several other unusual 24-ethyl sterols, including fucosterol (24*E*-ethylidene-cholesterol) and 24-ethyl-desmosterol. These results indicated the existence of multiple transmethylation pathways, operating at both the first and second stage of C-24 alkylation. The detection of 24(28)-methylene-25-methyl-cholesterol and 24-methyl-desmosterol indicated that the two sterols might be in a unique precursor–product relationship, as

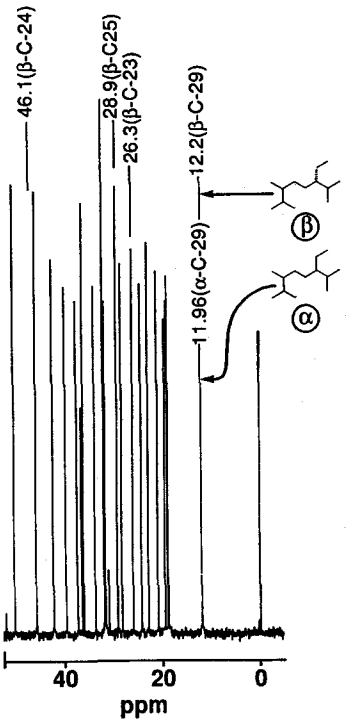


FIG. 5. ^{13}C Nuclear magnetic resonance spectrum of sitosterol isolated from corn sheaths. The relevant chemical shift for C-29 that characterize sitosterol and 24-episitosterol (clionasterol) is highlighted in the figure.

shown elsewhere in Kalinowska *et al.* (28). The detection of fucosterol and clereosterol [24β-ethyl-cholesta-5,25(27)-dienol] in corn along with isofucosterol was significant in terms of sterol phylogenesis, since only isofucosterol was thought to be synthesized by highly advanced plants such as corn (28,47). Rendell *et al.* (24) found that corn "sitosterol" contained a few percent of its 24-epimer as judged by ^1H NMR. Our samples of sitosterol were analyzed by ^{13}C NMR, a spectroscopic technique that is better suited than ^1H NMR to distinguish the chiral purity of C-24 alkyl sterols, because of the signal-to-noise ratio. All of our sitosterol samples were found to be epimerically pure 24α-ethyl-cholesterol. From the 4,4-dimethyl sterol fraction, we isolated two pentacyclic triterpenoids, α- and β-amyrin along with cycloartenol and 24-alkylated cycloartenols. For kinetic studies on sterol biosynthesis, we developed HPLC methods to separate the amyrins from cycloartenol and 24-alkylated cycloartenols (see Materials and Methods, and Table 1).

Changes in sterol content and distribution. Figure 7 shows that in the first eight days following seed germination, total sterol content of roots and shoots increased in parallel with increased body length. The rate of daily sterol production was found to be *ca.* 4 to 5 μg/day. Koops and Groeneveld (33) reported similar rates of sterol production for etiolated *Euphorbia lathyris* seedlings. The results indicated active sterol synthesis in the seedling to meet the demands of new plant growth. There was no significant change in the sterol profile of the seed during imbibition (sterol content remained at *ca.*

TABLE 2
Sterol Composition of *Zea mays*[a]

Sterol[b]	Structure	MS (M^+)	TLC (Rf)	Reference[c]	Plant source[d]
Cycloartenol[e]	1B	426	0.29	2,3,7,9–12,16,18–21,23,TL	st, c, g, r, sh, b, p
24(28)-Methylene-cycloartanol	1G	440	0.29	2,3,7,9–12,16,18–21,23,TL	st, c, g, r, sh, b, p
Cyclosadol	1E	440	0.29	7,16,18,20,TL	st, g, sh
Cyclolaudenol	1H	440	0.29	20	st
Cycloartanol*	1C	428	0.29	TL	sh
24-Methylcycloartanol	1J	442	0.29	2,7,TL	g
24(28)-Methyleneparkeol*	5G	440	0.29	TL	sh
α-Amyrin(triterpene)	19	426	0.29	2,7,9,10,12,18,20,TL	st, c, g, r, sh, b
β-Amyrin(triterpene)	20	426	0.29	2,3,7,9,10,12,18–20,23,TL	st, c, g, r, sh, b
4α,14α-Dimethylergosta-7,24(28)-dienol	9G	424	0.25	TL	st, c, g, r, sh, b
Lophenol	11C	400	0.25	7,11,12,TL	g, sh
24-Methylene-lophenol	11G	412	0.25	2,7,9–12,18,19,TL	c, g, r, i, sh, b, p
24-Methyl-lophenol	11J	414	0.25	7,12,TL	g, sh
24-Ethyl-lophenol	11S	428	0.25	7,12,TL	g
Cycloeucalenol	7G	426	0.25	2,7,9–12,18,19,23,TL	c, g, r, sh
Obtusifoliol	8G	426	0.25	2,7,9–11,18,19,23,TL	c, g, r, sh, b, p
Dihydroobtusifoliol*	8J	428	0.25	TL	sh
31-Norlanosterol*	8B	412	0.25	TL	sh
4α-Methylergosta-8,24(28)-dienol*	10G	412	0.25	TL	b
4α-Methylergosta-7(*E*)-23-dienol	11E	412	0.25	7,18,TL	c, g, sh
4α-Methylergosta-7(*Z*)-23-dienol*	11F	412	0.25	TL	sh
Citrastadienol	11P	426	0.25	2,7,9,10,18,19,TL	c, g, r, sh, b
Isocitrastadienol*	11Q	426	0.25	TL	sh
4α,14α-Dimethyl-ergosta-8(*E*)-23-dienol	8E	426	0.25	18,TL	c, sh
4α,14α-Dimethyl-ergosta-8(*Z*)-23-dienol*	8F	426	0.25	TL	sh
4α,14α-Dimethyl-24-ethyl-cholest-8-enol*	8S	442	0.25	TL	sh
4α,14α-Dimethyl-9,19-cycloergost-23-enol	7E	426	0.25	18,TL	c, sh
4α-Methyl-cholesta-8(9),14(15),24(28)-trienol*	12G	410	0.25	TL	sh
Cholesta-5,22-dienol*	13A	384	0.18	TL	sh
Cholest-7-enol*	14C	386	0.16	TL	b
Cholest-8(9)-enol*	15C	386	0.18	TL	b
Cholesterol	13C	386	0.18	1,3,4,6,7,9–15,18,20–23,TL	st, c, g, sh, b, p
Cholestanol	16C	388	0.16	20,21	st
Brassicasterol	13A	398	0.18	1,20,21,TL	st, sh
24-Methylene-cholesterol	13G	398	0.18	7,14,18,20,21,TL	st, c, g, sh, b, r, t, p
Ergosta-5(*E*)-23-dienol	13E	398	0.18	7,17,18,20,TL	st, c, g, sh, b, r
Codisterol	13H	398	0.18	20,TL	st, sh
Ergosta-7(*E*)-23-dienol	14E	398	0.16	17,18,20,TL	st, c, sh
24 Methylene-cholest-7-enol	14G	398	0.16	18,20,TL	st, c, sh, p
24-Methylene-zymosterol	15G	398	0.18	TL	p
Campesterol	13J	400	0.18	1,3–15,17–23,TL	st, c, g, sh, b, r, t, p
24-Epicampesterol	13I	400	0.18	7,18,20,TL	st, c, g, sh, b, r, p
Ergost-(*E*)-23-enol**	16E	400	0.16	TL	sh
14α-Methyl-cholest-7-enol*	18C	400	0.16	TL	sh
Ergost-7-enol	14J	400	0.16	18,20	st, c
Ergost-8(9)-enol*	15J	400	0.18	TL	sh
Ergostanol	16J	402	0.16	9,20,21,TL	st, c, sh
24β-Ethylcholesta-5,22,25-trienol	13L	410	0.18	TL	sh
14α-Methylergosta-8,25-dienol*	17H	412	0.18	TL	sh
14α-Methylergosta-8,24(28)-dienol*	17G	412	0.18	TL	sh
Stigmasta-7,25-dienol	14M	412	0.16	TL	sh
Stigmasta-8,25-dienol*	15M	412	0.18	TL	sh
24β-Ethyl-cholesta-5,25-dienol	13M	412	0.18	20,TL	st, sh
Stigmasta-5,23-dienol	13O	412	0.18	TL	sh
Fucosterol	13P	412	0.18	7,20,TL	st, g, sh
Isofucosterol	13Q	412	0.18	1,6,7,12,14,17–21,23,TL	st, c, g, sh, b, r, t, p
24-Ethylcholesta-5,24(25)-dienol	13R	412	0.18	20,TL	st, sh
Avenasterol	14Q	412	0.16	1,5,18,20,21,TL	st, c, sh
25-Methyl-24-methylene-cholesterol*	13K	412	0.18	TL	sh
Stigmasterol	13N	412	0.18	1,3–15,17–23,TL	st, c, g, sh, b, r, t, p
Stigmast-7-enol	14N	412	0.16	1,3–6,18	c
Stigmast-22-enol	16N	414	0.16	9,20,21,TL	st, sh
14α-methylergost-8(9)-enol	17J	414	0.18	TL	sh
Sitosterol	13S	414	0.18	1,3–15,17–23,TL	st, c, g, sh, b, r, t, p
Stigmastanol	16S	416	0.16	20,21,TL	st, sh

[a]MS, mass spectrometry; TLC, thin-layer chromatography. [b]*New corn sterols; **new natural sterol. [c]TL, this laboratory. [d]st, Shoot; c, coleoptile; g, germ oil; sh, sheath; b, blade; r, root; i, inflorescence; t, tassel; p, pollen. [e]Either trivial or systematic name is given.

TABLE 3
^{1}H NMR Spectral Values of *Zea mays* Sterols[a]

Sterol	H-18	H-19	H-21	H-26	H-27	H-28	H-29	H-30	H-31	H-32	Olefinic proton
Cycloartenol	0.966(*s*)	a-0.554(*d*) b-0.333(*d*)	0.881(*d*)	1.687(*s*)	1.607(*s*)	—	—	0.967(*s*)	0.810(*s*)	0.890(*s*)	24H-4.690(*dd*)
24(28)-Methylene-cycloartanol	0.968(*s*)	0.555(*d*) 0.336(*d*)	0.896(*d*)	1.079(*d*)	1.070(*d*)	4.692(*d*)	—	0.968(*s*)	0.811(*s*)	0.899(*s*)	—
Cyclosadol	0.969(*s*)	0.555(*d*) 0.331(*d*)	0.836(*d*)	0.988(*d*)	0.988(*d*)	1.552(*s*)	—	0.969(*s*)	0.810(*s*)	0.895(*s*)	—
Obtusifoliol	0.713(*s*)	0.972(*s*)	0.930(*d*)	1.032(*d*)	1.029(*d*)	4.670(*d*)	—	—	0.998(*d*)	0.887(*s*)	—
24(28)-Methylene-lophenol	0.538(*s*)	0.828(*s*)	0.956(*d*)	1.029(*d*)	1.024(*d*)	4.682(*d*)	—	—	0.989(*d*)	—	7H-5.196(*brd*)
4α-Methylergosta-7(*E*)-23-dienol	0.539(*s*)	0.827(*s*)	0.897(*d*)	0.986(*d*)	0.986(*d*)	1.545(*d*)	—	—	—	—	7H-5.191(*brd*) 23H-5.145(*brd*)
Citrostadienol	0.536(*s*)	0.827(*s*)	0.952(*d*)	0.976(*d*)	0.976(*d*)	5.109(*q*)	1.571(*d*)	—	0.989(*d*)	—	7H-5.183(*brd*)
Cholesterol	0.675(*s*)	1.006(*s*)	0.911(*d*)	0.862(*d*)	0.860(*d*)	—	—	—	—	—	6H-5.351(*brd*)
24(28)-Methylene-cholesterol	0.683(*s*)	1.011(*s*)	0.948(*d*)	1.029(*d*)	1.023(*d*)	4.686(*d*)	—	—	—	—	6H-5.355(*brd*)
Ergosta-5(*E*)-23-dienol	0.686(*s*)	1.009(*s*)	0.889(*d*)	0.986(*d*)	0.986(*d*)	1.545(*s*)	—	—	—	—	6H-5.356(*brd*) 23H-5.139(*t*)
24-Methylenecholest-7-enol	0.540(*s*)	0.797(*s*)	0.954(*d*)	1.028(*d*)	1.023(*d*)	4.698(*d*)	—	—	—	—	7H-5.184(*brd*)
24-Methylene-zymosterol	0.613(*s*)	0.951(*s*)	0.964(*d*)	1.028(*d*)	1.025(*d*)	4.696(*d*)	—	—	—	—	—
Ergosta-(*E*)-23-enol	0.655(*s*)	0.803(*s*)	0.871(*d*)	0.984(*d*)	0.984(*d*)	1.542(*s*)	—	—	—	—	23H-5.131(*t*)
Campesterol	0.679(*s*)	1.009(*s*)	0.910(*d*)	0.851(*d*)	0.802(*d*)	0.774(*d*)	—	—	—	—	6H-5.355(*brd*)
24-Epicampesterol	0.678(*s*)	1.010(*s*)	0.918(*d*)	0.781(*d*)	0.855(*d*)	0.775(*d*)	—	—	—	—	6H-5.355(*brd*)
Isofucosterol	0.682(*s*)	1.010(*s*)	0.946(*d*)	0.976(*d*)	0.976(*d*)	5.107(*q*)	1.597(*d*)	—	—	—	6H-5.358(*brd*)
24-Ethylcholesta-5,24(25)-dienol	0.686(*s*)	1.011(*s*)	0.919(*d*)	1.631(*d*)	1.631(*d*)	—	0.958(t)				6H-5.355(*brd*)
24β-Ethylcholesta-5,25-dienol	0.674(*s*)	1.010(*s*)	0.908(*d*)	1.568(*d*)	4.688(*s*)	—	0.803(*t*)	—	—	—	6H-5.357(*brd*)
Stigmasterol	0.701(*s*)	1.012(*s*)	1.022(*d*)	0.847(*d*)	0.801(*d*)	—	0.807(*t*)	—	—	—	6H-5.352(*brd*)
Sitosterol	0.679(*s*)	1.009(*s*)	0.920(*d*)	0.834(*d*)	0.812(*d*)	—	0.845(*t*)	—	—	—	6H-5.353(*brd*)

[a]NMR, nuclear magnetic resonance; chemical shifts are given in ppm from tetramethylsilane; *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *brd*, broad; *J* values vary from 5 to 7 Hz.

118 μg/seed for day 1 following imbibition). Kemp *et al.* (8) reported that during the first two weeks of corn seedling development, sterol increased in the shoot and root, as we reported here. Kemp *et al.* (8) found that the sterol composition of the shoot did not change qualitatively during seedling maturation whereas the root sterol composition did. In the root, the sitosterol levels decreased whereas stigmasterol levels increased. Alternatively, in our study we observed that the sterol composition changed in subtle ways qualitatively and quantitatively in the root and shoot (notably when the shoot is separated into blade and sheath) during seedling development. Table 4 shows the sitosterol/stigmasterol ratio and 24α–/24β-methyl-cholesterol ratio changes during physiological aging. The level of ergosta-5,23-dienol increased during development of dark-grown coleoptiles (sheaths). Actively growing tissues, such as root caps, tissue cultures, and blades, produced mainly 24-ethyl-sterols, and sitosterol predominated the sterol mixture. Pollen sterols were unusual both qualitatively and quantitatively. Pollen accumulated 24(28)-methylene-sterols, including the Δ^7-sterols, 24(28)-methylene-lophenol, and cholesta-7,24(28)-dienol. Of the plant parts studied, pollen produced the most sterol (3,904 μg sterol/g fresh weight) whereas the three-day shoot possessed the least sterol (94 μg sterol/g fresh weight).

Relative rates of sterol biosynthesis and transformation. Table 5 shows the incorporation of [2-^{3}H]acetate into sheath and blade sterols after 24-h incubation. When the blade 4,4-desmethyl-sterol fraction was purified into individual sterols by HPLC, the bulk of the radioactivity was found to be with six Δ^5-sterols: as percent of total activity, ^{3}H was associated with 24(28)-methylene-cholesterol (9%), campesterol (4%), 24-epicampesterol (5%), isofucosterol (28%), sitosterol (43%), and stigmasterol (7%). When the sheath 4,4-desmethyl-sterols were purified by HPLC (Fig. 8), it was clear that neither stigmasterol, sitosterol, nor 24-epicampesterol incorporated significant label from [2-^{3}H]acetate whereas 24(28)-methylene-cholesterol, ergosta-5,23-dienol, and campesterol were labelled significantly. These results suggested that sheath and blade produce many of the same sterols, but that their production rates differ.

Figure 9 shows that [2-^{14}C]mevalonic acid uptake was linear up to about 10 h. There was much greater incorporation of [2-^{14}C]mevalonic acid into 4,4-desmethyl-sterols in blades than in sheaths. These results were consistent with the [2-^{3}H]acetate feeds to eight-day seedlings. From [2-^{14}C]-mevalonic acid experiment, the relative rate of 4,4-desmethyl-sterol synthesis in the blade was found to be *ca.* 1.0 pmol/h/100 shoots and the ratio of relative rates of synthesis of 24-methyl- and 24-ethyl-sterols about 4:1, which is in good agreement with the data observed by Rendell *et al.* (24), who studied kinetics of sterol synthesis in etiolated corn shoots. Scheid *et al.* (18) studied the kinetics of sterol biosyn-

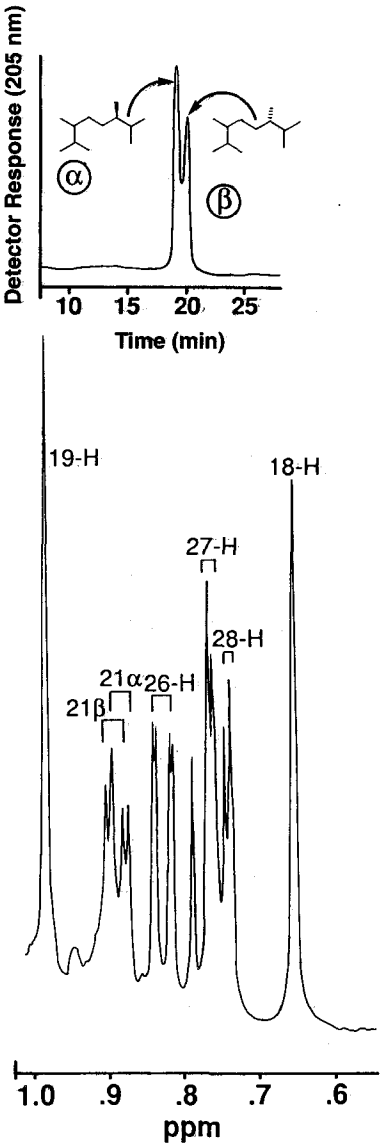


FIG. 6. High-performance liquid chromatography chromatogram [sample chromatographed on a TSK gel column (Toya Soda Manufacturing Co., Tokyo, Japan)] (top) and ^{1}H nuclear magnetic resonance spectrum (300 MHz) (bottom) of a diastereoisomeric mixture of 24-methyl-cholesterol isolated from eight-day corn sheaths.

thesis in the sheath of etiolated corn seedlings. They found that neither sitosterol, stigmasterol, nor 24-methyl-sterols were labeled significantly from incubation with [2-^{14}C]acetate, whereas ^{14}C label was incorporated into 24(28)-methylene-cholesterol and ergosta-5,23-dienol. Rendell *et al.* (24) reported that 24-ethyl-sterols were labeled significantly in etiolated corn shoots.

We were puzzled by the different results. However, as our results on the separated anatomical parts, i.e., sheaths and blades, clearly show, the blade is biosynthetically more active in producing sterols than is sheath. Thus, Rendell *et al.* (24) seem to have quantified blade sterol synthesis in the shoots.

Pulse chase experiments. Figure 10 shows an experiment that involved an eight-hour equilibrium-labeling period using [2-^{14}C]mevalonic acid as substrate followed by a period for up to three hours when mevalonic acid was chased into cycloartenol. Table 6 shows the product analysis upon chasing the label into 4,4-desmethyl-sterols. Regardless of whether the tissue was blade or sheath, cycloartenol was turned over rapidly: 24 pmol/h/mg protein during the chase whereas the turnover rates of the 4,4-desmethyl-sterols were an order of magnitude lower than those of cycloartenol (data not shown). There were no significant differences in cycloartenol synthesis in blades and sheaths during the three-hour chase, whereas significant differences were found in the relative rates of individual sterol end products. As shown in Table 6, sitosterol was synthesized slowly whereas ergosta-5,23-dienol was synthesized rapidly. Stigmasterol synthesis was very low, as reported by others studying corn (24), and by us studying sorghum (30). Stigmastanol, which appears just after sitosterol in HPLC, was not synthesized actively, in contrast to the findings of Misso and Goad (20) who reported active synthesis of stanols. The high rate of 24(28)-methylene-cholesterol turnover shown in Table 6 is consistent with its playing a role as an intermediate rather than an end product of cycloartenol transformation to Δ^5-sterol end products (31). To explain the differences in the kinetics of sitosterol and stigmasterol synthesis, Rendell *et al.* (24) proposed a two-pool model. This model might also explain the labeling kinetics of stanols. However, the model will not adequately explain the differences in 24-methyl- and 24-ethyl-sterol synthesis. Our hypothesis is that cycloartenol was channeled into two C-24 alkylation pathways, the $\Delta^{24(28)}$-methylene and Δ^{23}-methyl pathway. The sheath operated both pathways whereas the blade operated only the $\Delta^{24(28)}$-methylene pathway. We assume differences in rates of Δ^5-sterol synthesis in sheaths and blades are not based on differences in carbon flow from mevalonic acid metabolism, because cycloartenol synthesis was equally active in both tissues. We hypothesize that the rate of Δ^5-sterol synthesis is controlled by cycloartenol turnover, which is controlled by the activity of the C-24 methyl transferase (35).

At this point we thought the blades and sheaths synthesized most of the sterol present in the tissue. However, when we calculated the relative rate of sterol synthesis from the data obtained in Figure 6 to be 30 nmol sterol produced/h/100 shoots, we concluded that there was a discrepancy between the natural product and biochemical determinations in estimating rates of sterol production. There was obviously too little sterol synthesized from exogenous acetate or mevalonate by the Bloch-Cornforth pathway to account for the level of sterol actually accumulating in the maturing seedlings. The question arose whether the sterol present in early maturing shoots was derived partly from *de novo* synthesis of some alternative pathway (e.g., the Popják shunt), or from translocation of sterol stored in the seed, or both. To answer this question, additional experiments were needed, as discussed next.

KIE. Table 5 shows the incorporation of label from [2-^{14}C]mevalonic acid and [2-^{14}C]acetic acid into the nonsaponifiable lipid fraction and into sterol fractions purified by

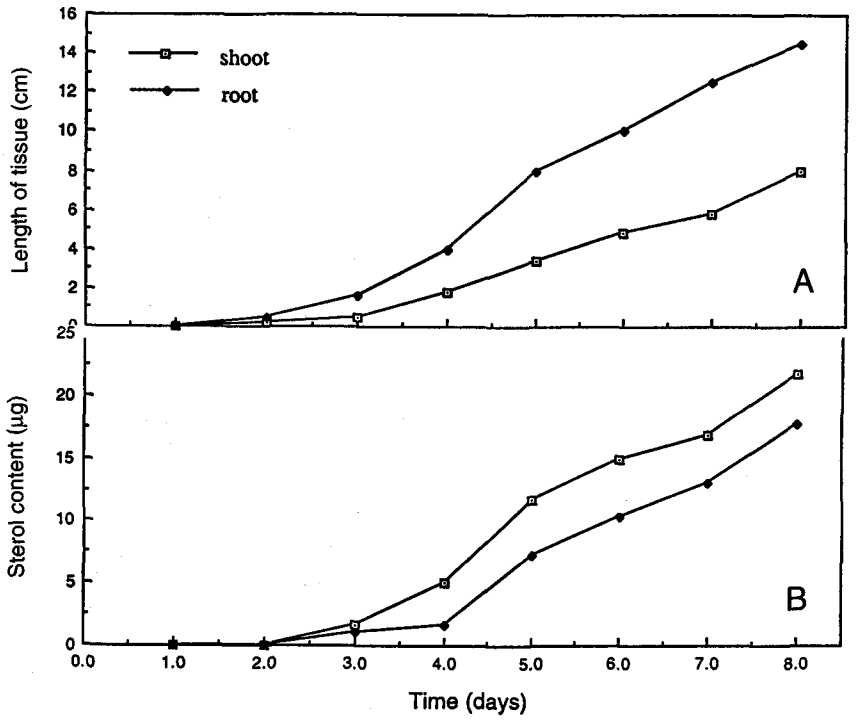


FIG. 7. A: Plot of root and shoot growth and B: changes in sterol content relative to time of seedling maturation. The day one value is the time following imbibition of water.

TLC. Whereas label from mevalonic acid was incorporated into 4-desmethyl-sterols, label from acetic acid was not. The results proved that seeds do not synthesize sterols *de novo* from acetate. To confirm further that the mevalonate to sterol pathway was not active in germinating seeds, corn seeds were imbibed with deuterated water. The seeds were slow to germinate, but after ten days, the seeds developed shoots of approximate length to three-day germinated plants incubated with normal water. The GLC/MS of the seed sterols confirmed that none were labeled with deuterium.

The three-day shoots from normal water germinated seeds were found to possess five main sterols by GLC. They were, based on their retention times in GLC (Fig. 11), ergosta-

TABLE 4
Sterol Composition of Corn Tissues[a,b]

Sterol	Structure	Tissue culture	Three-day shoot	Four-day blade	Four-day sheath	Four-day root tip	Four-day root base	Eight-day sheath	Eight-day blade	Eight-day root	Dry seed	Dry pollen
Cycloartenol	1B	0.8	0.6	0.5	1.7	1.2	0.9	0.7	0.4	1.1	0.6	1.7
24(28)-Methylene-cycloartanol	1G	5.1	0.5	0.3	0.5	1	1.3	0.1	0.2	0.3	1.4	1.8
Cyclosadol	1E	N.D.	0.04	N.D.	0.4	N.D.	N.D.	0.06	N.D.	N.D.	trace	N.D.
Obtusifoliol	8G	0.5	0.4	0.2	0.8	1.3	0.4	0.3	0.2	0.4	1.2	1.9
24(28)-Methylene-lophenol	11G	0.3	0.8	0.3	0.8	1.4	0.6	0.4	0.2	0.3	1	26.5
Citrastadienol	11Q	2.7	0.3	0.2	0.3	1.6	0.3	0.1	0.1	0.3	0.5	N.D.
Cholesterol	13C	0.1	1	trace	1.2	trace	1.3	1.2	0.2	0.6	trace	trace
24(28)-Methylene-cholesterol	13G	N.D.	0.8	0.5	0.8	0.8	0.3	1.4	0.4	0.4	1.3	17.7
Ergosta-5,(E)-23-dienol	13E	N.D.	8.4	0.04	11.4	N.D.	N.D.	10.9	0.04	N.D.	0.3	N.D.
24-Methylene-cholest-7-enol	14G	N.D.	trace	trace	0.2	trace	trace	0.3	trace	trace	trace	44.2
Isofucosterol	13Q	1.8	8	10	4.1	16.1	4	1.9	3.6	9	9	1.5
Campesterol	13J	4.1	6	5.6	4.4	8.1	8.1	9.1	7.9	11	9.3	0.3
24-Epicampesterol	13I	3.3	8.4	6.1	6.1	8.8	9.1	12	8.6	11.6	13.9	0.2
Sitosterol	13S	43.3	37.3	61.9	28.9	30.8	21.6	20	57.7	18.9	52.5	0.9
Stigmasterol	13N	36.6	25.4	13.7	38.1	27.2	50.5	40.5	19.7	50.2	7.3	0.9
Total sterol (µg/g fresh weight)		140.2	94.1	245.8	199.8	295.7	118.6	72.6	190.3	111.6	590.7	3904.5

[a]As percent of total sterol; several other minor sterols were detected as shown in Table 2. [b]Seed and pollen are in µg/g dry weight; N.D., not detected.

TABLE 5
Incorporation of Labeled Intermediates into *Zea mays* Sterols

	Total activity (dpm × 10^5)									
	Blade[a]			Sheath[a]					^2H_2O	^2H_2O
Fraction	A	B	C	A	B	C	Seed[b]	Seed[c]	Shoot[d]	Seed[d]
NSF[e]	347	15.3	9.1	110	12.3	9.9	8.1	1.0	NA[f]	NA
4,4-Dimethyl-sterol[g]	44.6	2.5	1.0	29.8	3.1	2.4	0.4	0.1	L(80%)	NL[f]
4-Monomethyl-sterol	29.8	1.2	0.4	21.4	1.7	0.4	0.7	0.1	L(66%)	NL
4-Desmethyl-sterol	56.6	5.4	0.8	6.6	1.7	0.4	0.3	NL	L(30%)	NL

[a]A: Incubated fifty seedlings (eight-day) with 50 μCi of [2-^{3}H]acetate for 24 h; B: incubated fifty seedlings (eight-day) in phosphate buffer with 5 μCi of [2-^{14}C]mevalonate for 8 h; C: incubated fifty seedlings (eight-day) in water with 4 μCi of [2-^{14}C]mevalonate for 10 h.
[b]Imbibed twenty seeds with 5 μCi of [2-^{14}C]mevalonate for 72 h (shoot removed).
[c]Imbibed twenty seeds with 5 μCi of [2-^{14}C]acetate for 72 h (shoot removed).
[d]Germinated 2,000 seeds in 400 mL ^2H_2O for ten days (shoot dissected from seed and examined separately). Data in parentheses represent percentage of total sterol in that fraction labelled with deuterium.
[e]NSF, nonsaponifiable lipid fraction.
[f]NA, not applicable; NL, not labeled.
[g]Fraction from thin-layer chromatography plate corresponding to the degree of substitution at C-4 of sterols. The 4,4-dimethyl-sterol band contains pentacyclic triterpenoids and long-chain fatty alcohols.

5,23(24)-dienol, 24-methyl-cholesterol, stigmasterol, sitosterol, and isofucosterol. The shoot sterol composition from seeds germinated in D_2O was different from the sterol composition of seeds germinated in H_2O (Fig. 11). A new peak appeared in the GLC corresponding to the relative retention time, measured relative to cholesterol (RRT_c), of stigmasta-5,23(24)-enol, and the peak for isofucosterol disappeared. A second novel compound, which was not readily evident in the GLC chromatogram of 4-desmethyl-sterols from the D_2O-treated shoots, was detected during HPLC fractionation of the 4-desmethyl-sterol mixture. Based on HPLC, the two new sterols represented about 30% of the total sterol mixture of the shoot. Shoot 4-desmethyl-sterols from the D_2O treatment were examined by GLC/MS. Two of the sterols were found to be labeled with deuterium, ergosta-5,23(24)-dienol and stigmasta-5,23(24)-dienol, whereas the other sterols were not labeled, e.g., sitosterol (Fig. 12). The apparent molecular weights of the two labeled sterols, M^+ 446 and 432 amu, respectively, indicated that 34 hydrogens were introduced into the sterol molecule from deuterium (in the form of deuterium introduced from NADPH and medium in the form of H^+). This number contrasts with the value of *ca.* 24 hydrogens previously hypothesized (48,49) as the number of hydrogens normally introduced into phytosterol synthesis (assuming complete equilibration of NADPH with D_2O) (48). The 14 mass unit difference between ergosta-5,23-dienol and stigmast-5,23-dienol indicated that the methyl on *S*-adenosyl-L-methionine was not labeled with deuterium, and therefore the amino acid must have been translocated along with sterols from the seed to the developing shoot. We were concerned with the character of the mass spectra of the deuterated sterols obtained by the GLC/MS (spectra obtained on a table top HP model GLC/MS). Therefore, we prepared 24,24-dideuterated 24-methyl-cholesterol by deuteration and 24-methyl-cholesterol by hydrogenation from 24-methyl-desmosterol, as described (50). As shown in Figure 13, protium-deuterium exchange occurred in GLC that affected the distribution of ions in the high mass region. The sample of deuterated 24-methyl-cholesterol was examined by ^1H and ^{13}C NMR and found to incorporate deuterium in place of hydrogen at C-24 and C-25 completely. We observed this exchange phenomenon with several other deuterated sterols (unpublished). We observed that as the number of deuterium atoms incorporated into the sterol increases, the molecular ion cluster becomes more prominent. It is important to note that the ions thought to represent endogenous substrate are due to protium/deuterium exchange resulting from the sample passing through chromatographic column which operates at high temperatures. The identities of the two deuterated sterols isolated from shoots were confirmed by purifying them by HPLC, and by then examining the pure sample by ^1H NMR. Both sterols showed NMR spectra consistent with the proposed structures (ergosta-5,23-dienol and stigmasta-5,23-dienol). Specifically, for the deuterated sample of ergosta-5,23-dienol, the spectra showed a characteristic olefinic proton resonating at δ 3.513 ppm (multiplet) for H-3 and at δ 5. 318 ppm for H-6 (doublet). The signal for the H-23 proton was observedat δ 5.109 ppm (partial triplet) (Fig. 14). There was also a small chemical shift and a change in the character of the signals for H-3, H-6, and H-23 in the ^1H NMR spectra of the deuterated compared to normal sample. The signals corresponding to H-3, H-6, and H-23 shifted due to an apparent β-deuterium isotope effect of (+) 0.016 ppm and (−) 0.014 ppm and (+) 0.005 ppm, respectively. The methyl region of the ^1H NMR spectra was complex. From the shoot 4,4-dimethyl TLC band, several sterols and triterpenes were isolated. Cycloartenol was labeled with deuterium (M^+ 460 amu) whereas none of the triterpenes nor 24(28)-methylene-cycloartanol were labeled. Lakshmann and Veech (48), studying cholesterol synthesis in the rat using ^3H_2O as the labeled intermediate, hypothesized on biosynthetic grounds (i.e., the Bloch-Cornforth pathway) that H-3, H-6, and H-23 on cholesterol should contain tritium.

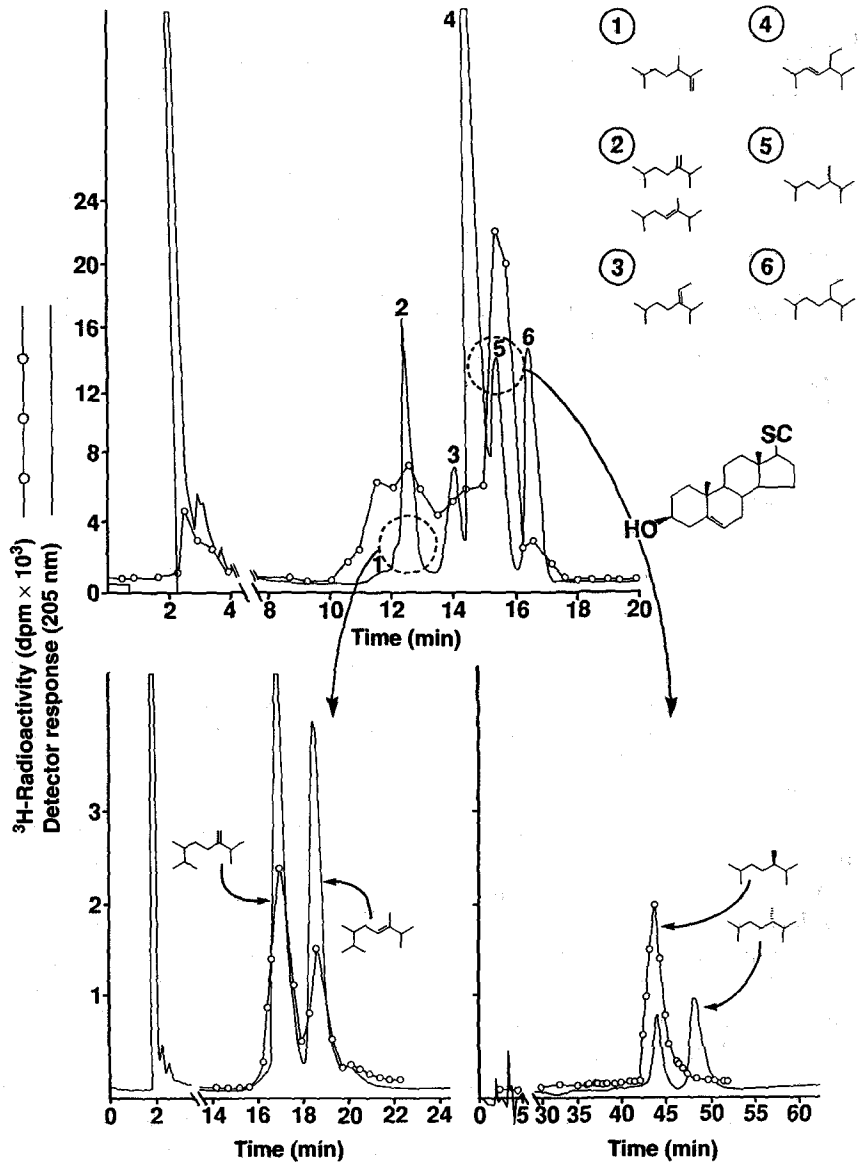


FIG. 8. Reversed-phase high-performance liquid chromatography (HPLC) (Zorbax C-18 column; Dupont, Boston, MA) mass and radioactivity analysis of the total 4-desmethyl-sterol fraction (top) isolated from sheaths of eight-day seedlings. Selected HPLC fractions, which were known to contain a mixture of sterols, were eluted from the Zorbax column and rechromatographed using a TSK gel C-18 column (Table 1), as shown in the bottom figure. See Figure 6 for company source.

The tritium at C-6 and C-23 should be derived from hydrogens derived from NADPH incorporated into mevalonic acid (48). The tritium incorporated into H-3 should be derived from reduction of a 3-keto sterol formed during the C-4 demethylation step of lanosterol. Our spectroscopic data on the deuterated Δ^{23}-alkyl-sterols do not support the Bloch-Cornforth labeling pattern for sterols. The signals in the ^{1}H NMR demonstrated that deuterated ergosta-5,23-dienol retained hydrogens at C-3, C-6, and C-23, which shows that mevalonic acid was not a precursor of the deuterated sterols.

The synthesis of deuterated ergosta-5,23-dienol and stigmasta-5,23-dienol suggests that the first and second methyl transfer at C-24 should be sensitive to a KIE. Most likely, there were originally a hydrogen and a deuterium at C-23, since the methyl cation has been shown to possess three hydrogen atoms. An induced KIE occurs when a primary deuterium isotope effect accelerates the rate of formation of one transmethylation product by suppressing the rate of formation of another transmethylation product. Isotopically sensitive branching describes the rate enhancement in the formation of one product of a multiple product enzyme caused by a reduction of the rate constant for the formation of a second

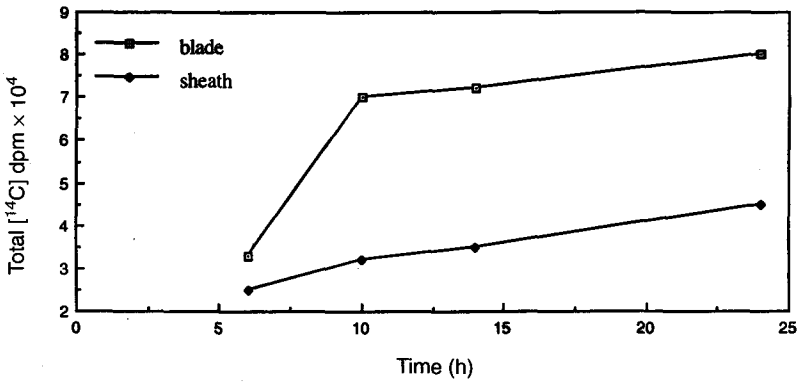


FIG. 9. Curves relating time of incubation of [2-^{14}C]mevalonic acid with the extent of [2-^{14}C]mevalonic acid incorporation into 4-desmethyl-sterols of blades and sheaths of eight-day seedlings (*cf.* Table 5).

product due to isotopic substitution in the substrate. Observations of this kind indicate that the two products arise from a common intermediate formed by the same enzyme (51). Goad *et al.* (52), studying sterol side-chain alkylation mechanisms in an alga, observed a KIE in the first and second transmethylation. From our data on the sterol composition of corn, we found that the plant produced several C-24 alkylated isomers, all of which may be described as originating mechanistically from the same reaction involving the same enzyme. The KIE operating during transmethylation favors abstraction of a proton from H-23 rather than from the incoming methyl cation (H-28) or from H-27. Assuming that a single C-24 methyltransferase mediates the production of several C-24 methylated alternatives, then the course and rate of methylated products formation should be affected, which is what was observed here. It follows that the deuterated Δ^{23}-24-alkyl-sterols were formed by disturbed methylation equilibria involving either cycloartenol giving rise to cyclosadol (first transmethylation) which is turned over to ergosta-5,23-dienol, or 24(28)-methylene-lophenol, giving rise to 4α-methyl-stigmasta-7,23-dienol which then is turned over to stigmasta-5,23-dienol.

In both deuterated samples of stigmasta-5,23-dienol and ergosta-5,23-dienol, H-6 and H-3 were labeled with hydrogen rather than deuterium. This was unexpected and suggests that the Bloch-Cornforth pathway (37), which may lead to the formation of the two deuterated 24-alkyl-sterols, might be downregulated during early shoot development. The unusual labeling pattern indicated either sugar (*via* pyruvate) or amino acid (possibly derived from pyruvate) was transformed into a 5-carbon molecule that entered into the isopentenoid pathway *via* the Popják shunt, effectively by-passing mevalonic acid. The 5-carbon molecule then could be phosphorylated and converted into isopentenyl pyrophosphate. Studies with stable isotopes are planned to shed light on this novel plant lipid pathway.

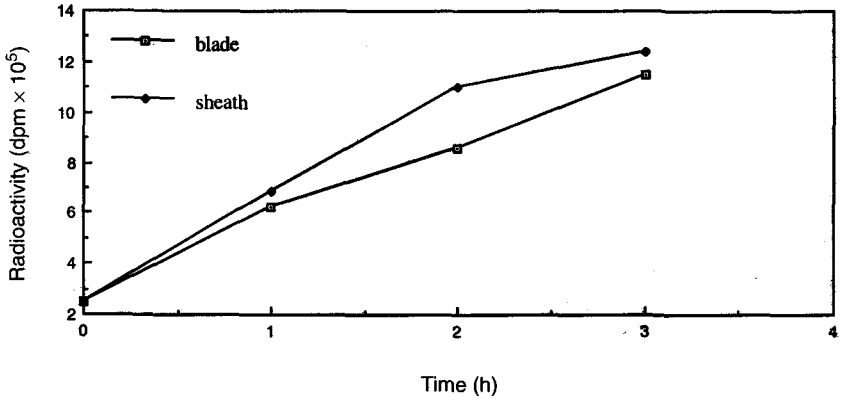


FIG. 10. Pulse-chase experiments which involved preequilibration of [2-^{14}C]mevalonic acid in a phosphate buffer with eight-day blades and sheaths, then chasing the label for up to 3 h into cycloartenol (purified by thin-layer chromatography and high-performance liquid chromatography) (*cf.* Table 6 for results of the chase of radioactivity into 4-desmethyl-sterol end products).

TABLE 6
Labeling Patterns in Sterols from Pulse Chase Experiment

	$\Delta^{24(28)}$-CH_2 Pathway						
	Blade (SA[a])		Sheath (SA[a])		Δ^{23}-24-CH_3 Pathway[b]		
						Sheath (SA[a])	
Sterol	8 h	11 h	8 h	11 h	Sterol	8 h	11 h
Cycloartenol	6.7×10^3	3.2×10^4	5.3×10^3	2.8×10^4			
24-Methylene-cycloartanol	4.4×10^3	2.1×10^4	4.1×10^3	2.1×10^4	Δ^{23}-24-Methyl cycloartanol	7.0×10^3	8.4×10^3
Obtusifoliol	1.1×10^4	3.2×10^4	1.5×10^4	4.4×10^4	Δ^{23}-24-Methyl obtusifoliol	3.3×10^3	9.6×10^3
24-Methylene-lophenol	6.2×10^3	8.8×10^3	1.3×10^3	1.9×10^3			
Citrastadienol	4.9×10^3	1.9×10^3	2.6×10^3	8.8×10^3	Δ^{23}-24-Methyl lophenol	2.2×10^3	4.4×10^3
24-Methylene-cholesterol	9.5×10^3	1.3×10^4	2.1×10^3	4.8×10^3			
Isofucosterol	5.8×10^3	7.0×10^3	3.5×10^3	7.2×10^3			
24-Methyl-cholesterol	2.8×10^2	3.9×10^2	5.3×10^1	2.3×10^2	Δ^{23}-24-Methyl cholesterol	5.3×10^3	1.2×10^4
Sitosterol	3.5×10^2	4.5×10^2	1.0×10^2	4.6×10^2			
Stigmasterol	1.2×10^1	1.3×10^1	4.7×10^0	6.1×10^0			

[a]SA, specific activity in dpm/nmol; 5.0 μCi of [2-^{14}C]mevalonic acid was supplied to fifty seedlings and incubated for 8 h. The seedlings were rinsed with water and placed into fresh buffer for 1, 2, and 3 h. Only the 8-h (zero-h chase time point) and 11-h (3-h chase time point) are reported.
[b]This pathway was not operational in blades.

CONCLUSIONS

It is well known that the activity of HMG–CoA reductase is important in regulating carbon flux from acetate to sterol. Nevertheless, for some time we have questioned whether HMG–CoA reductase is "the" rate-limiting enzyme in phytosterol transformation, a biochemistry which may be viewed as the "post-cycloartenol" pathway (32,36). We believe the search for the critical rate-limiting step in phytosterol transformation (which is distinct from steps that lead to cycloartenol production) should be directed to enzymes that act on the tetracyclic structure (36). In this study we discovered that the C-24 alkylation step was a rate-limiting step in sterol transformations. We define the start of the sterol pathway with the formation of cycloartenol (4,4-dimethyl-sterol) and ending with the formation of Δ^5-sterols (4-desmethyl-sterol) (36). Stanols, such as stigmastanol (sitostanol), might be considered the end products of cycloartenol metabolism. They are likely formed by conversion of the Δ^5,3-OH compound to a Δ^4,3-one, and reduced to produce the Δ^0-compound as shown chemically (53). Stanols, unlike Δ^5-sterols, do not accumulate in membranes (54); therefore by definition (36) they cannot be end products equivalent functionally to sitosterol. They either accumulate as ferculate esters in corn seeds (55) or form part of the surface wax (unpublished observations). We hypothesized that the steady-state concentration and structure of the end product are important in ways that go beyond their role as membrane inserts (36). In sunflower we found that the end product may feed back on the transformation process by binding competitively with other sterols that may be viewed as the natural (preferred) substrates (56,57). Thus it appeared that the precise outcome of cycloartenol transformation to 24-alkyl-sterols depended on substrate availability and structure of sterols. Additionally important are the corresponding rate constants (kinetic control) for binding and catalysis by the C-24 methyltransferase and thermodynamic control where product utilization promotes the different kinetically favored pathways. A similar pathway appears to be operating in corn.

As shown by our kinetic studies and confirmed by the sterol composition data of maturing seedlings, carbon from cycloartenol flows into sitosterol first. Then secondly, as tissues age, carbon flows into 24-methylene- and 24-methyl-sterols. The accumulation of 24-methyl-sterols in some tissues means the activity of the C-24 methyltransferase was affected. Figure 15 shows our hypothetical kinetically favored 24-alkylation pathway in actively growing tissues (albeit that involving active cell proliferation). There are important branch points in the pathway which are controlled by C-24 alkylation.The critical slow step, or the first committed step in the corn sterol pathway, appears to be methylation of cycloartenol. However, by the regulating of C-24 methyltransferase activity, e.g., of cycloartenol transmethylation, the kinetics of the pathway may be shifted from one that produces 24(28)-methylene-sterols giving rise to sitosterol to one which gives rise to Δ^{23},24-methyl-sterols, such as cyclosadol and ergosta-5,23-dienol. If there is sufficient preformed sterol available for transmethylation, as was the case in the developing shoot (*via* translocation from the seed), the second alkylation step may become rate-determining. Here enzyme activity may be regulated by conformational control resulting from structurally similar sterols competing for the same sites on the C-24 methyltransferase. The inability for the deuterated Δ^{23},24-alkyl sterols to be turned-over in the shoot may indicate that the side-chain reductase is regiospecific for the 24,25-bond or that a KIE was operating with respect to the 23,24-reductase.

The novel labeling pattern in the deuterated sterols isolated from the shoots of seeds germinated in D_2O water indicates that carbon flux into the isopentenoid pathway and into sterols

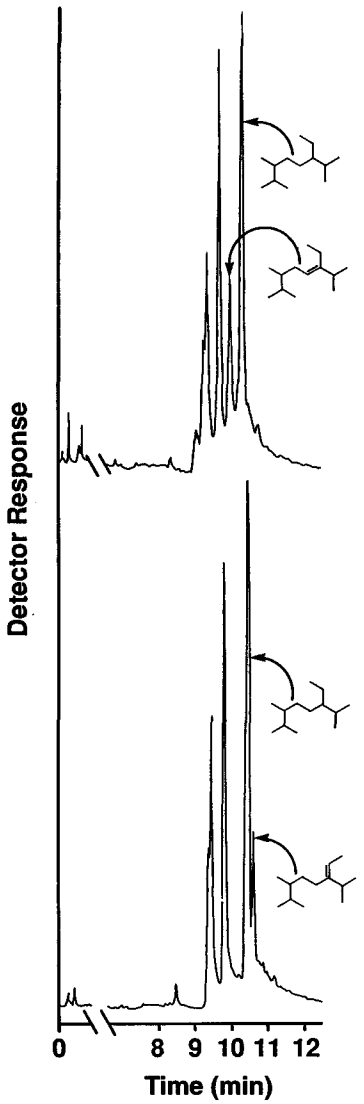


FIG. 11. Gas–liquid chromatographic (GLC) separation of 4-desmethylsterols from shoots grown on D_2O (top) or H_2O (bottom). The GLC column was a 15 m capillary HP-5 column operated isothermally at 245°C. The GLC column was different from the one used for routine sterol analysis described in the Results section.

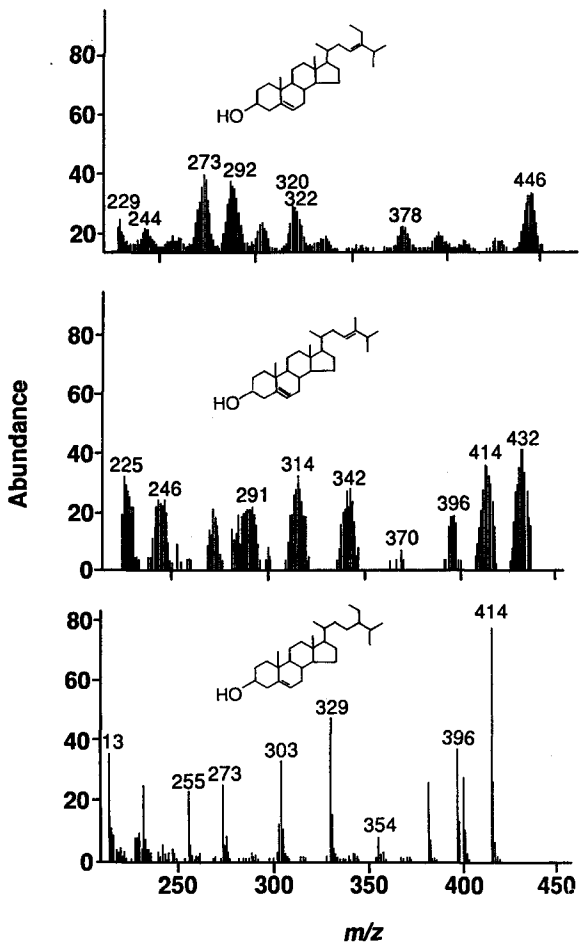


FIG. 12. Mass spectra of deuterated (top two spectra) and nondeuterated (bottom spectrum) sterol samples isolated from shoots incubated with D_2O and purified by high-performance liquid chromatography. Only the high mass region is reported.

may involve novel pathways that require participation of the Popják shunt.

ACKNOWLEDGMENT

This work was supported by a grant from the Asgrow Seed Company.

REFERENCES

1. Itoh, T., Tamura, T., and Matsumoto, T. (1973) *J. Am. Oil Chem. Soc. 50,* 122–125.
2. Itoh, T., Tamura, T., and Matsumoto, T. (1973) *J. Am. Oil Chem. Soc. 50,* 300–303.
3. Fedeli, E., and Mariani, C. (1974) *Riv. Ital. Sostan. Gra. 51,* 129–132.
4. Mannino, S., and Amelotti, G. (1975) *Riv. Ital. Sostan. Gra. 52,* 79–83.
5. Seher, A., and Vogel, H. (1976) *Fette, Seifen, Anstrichm. 78,* 301–305.
6. Prevot, A.F., and Morret, F.X. (1976) *Rev. Fr. Cor. Gras. 23,* 409–423.
7. Itoh, T., Shimizu, N., Tamura, T., and Matsumoto, T. (1981) *Phytochemistry 20,* 1315–1356.
8. Kemp, R.J., Goad, L.J., and Mercer, E.I. (1967) *Phytochemistry 6,* 1609–1615.
9. Kemp, R.J., and Mercer, E.I. (1968) *Biochem. J. 110,* 111–118.
10. Kemp, R.J., and Mercer, E.I. (1968) *Biochem. J. 110,* 119–125.
11. Kemp, R.J., Hamman, S.A., Goad, L.J., and Goodwin, T.W. (1968) *Phytochemistry 7,* 447–450.
12. Rohmer, M., Ourisson, G., and Brandt, R.D. (1972) *Eur. J. Biochem. 31,* 172–179.
13. Hartmann, H.A., Normand, G., and Benveniste, P. (1975) *Plant Sci. Lett. 5,* 287–292.
14. Knight, B.A., and Smith, A.R. (1976) *Planta 133,* 89–93.
15. Comita, J.J., and Klosterman, M.J. (1976) *Phytochemistry 15,* 917–920.
16. Pinhas, H. (1969) *Bull. Soc. Chim. Fr. 6,* 2037–2039.
17. Scheid, F., and Benveniste, P. (1979) *Phytochemistry 18,* 1207–1209.

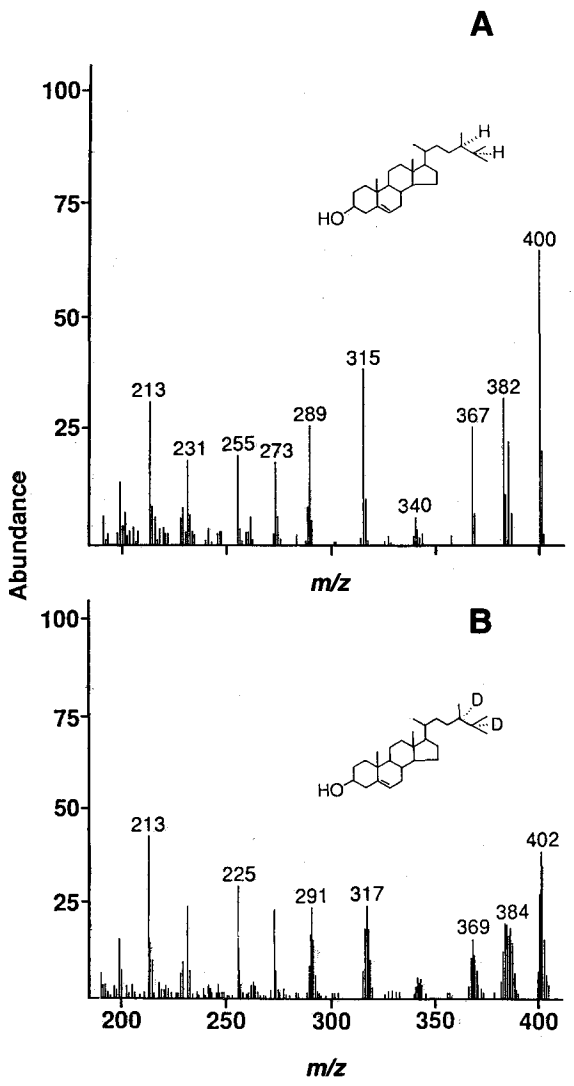


FIG. 13. A: Mass spectra of 24-methyl-cholesterol prepared synthetically by hydrogenation of 24-methyl-desmosterol using either hydrogen, or B: deuterium gas.

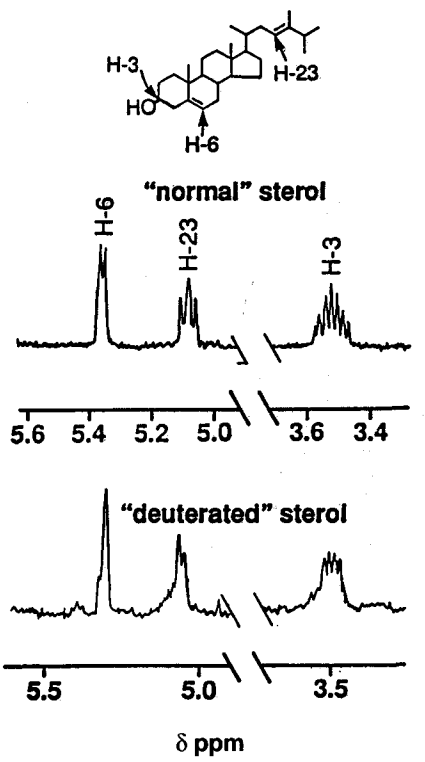


FIG. 14. Partial ^1H nuclear magnetic resonance spectra (300 MHz) of ergosta-5,23-dienol isolated from shoots incubated with H_2O (top) and D_2O (botttom). Only selected diagnostic chemical shifts are reported.

18. Scheid, F., Rohmer, M., and Benveniste, P. (1982) *Phytochemistry 21*, 1959–1967.
19. Bladocha, M., and Benveniste, P. (1983) *Plant Physiol. 71*, 756–762.
20. Misso, N.L.A., and Goad, L.J. (1984) *Phytochemistry 23*, 73–82.
21. Chitwood, D.J., Hutzell, P.A., and Lusby, W.R. (1985) *J. Nematol. 17*, 64–68.
22. Navari-Izzo, F.F., Izzo, R., Bottazzi, F., and Ranieri, A. (1988) *Phytochemistry 27*, 3109–3115.
23. Grandmougin, A., Bouvier-nave, P., Ullmann, P., Benveniste, P., and Hartmann, M. (1989) *Plant Physiol. 90*, 591–597.
24. Rendell, N., Misso, N.L.A., and Goad, L.J. (1986) *Lipids 21*, 63–68.
25. Benveniste, P. (1986) *Annu. Rev. Plant Physiol. 37*, 275–308.
26. Bach, T.J. (1985) *Lipids 21*, 82–88.
27. Nes, W.D., and Bach, T.J. (1985) *Proc. Royal Soc. Lond. B. 225*, 425–444.
28. Kalinowska, M., Nes, W.D., Crumley, F.G., and Nes, W.D. (1990) *Phytochemistry 29*, 3427–3434.
29. Nes, W.D., and Schmidt, J.O. (1988) *Phytochemistry 276*, 1705–1708.
30. Heupel, R.C., Sauvaire, Y., Le, P.H., Parish, E.J., and Nes, W.D. (1986) *Lipids 21*, 69–75.
31. Nes, W.D. (1990) *Rec. Adv. Phytochem. 24*, 283–327.
32. Nes, W.D., Janssen, G.G., Norton, R.A., Kalinowska, M., Crumley, F.G., Tal, B., Bergenstrahle, A., and Josson, L. (1991) *Biochem. Biophys. Res. Commun. 177*, 566–574.
33. Koops, A.J., and Groeneveld, H.W. (1991) *J. Plant Physiol. 138*, 142–149.
34. Rohmer, M., Knani, M., Simonin, P., Sutter, B., and Sahm, H. (1993) *Biochem. J. 295*, 517–524.
35. Nes, W.D., Parker, S.R., Crumley, F.G., and Ross, S.A. (1993) *Lipid Metabolism in Plants* (Moore, T.S., ed.) pp. 389–426, CRC Press, Boca Raton.
36. Parker, S.R., and Nes, W.D. (1992) *ACS Symp. Ser. 497*, 110–145.
37. Nes, W.R., and Mckean, M.L. (1977) *Biochemistry of Steroids and Other Isopentenoids*, pp. 100–535, University Park Press, Baltimore.
38. Moss, G.P. (1989) *Eur. J. Biochem. 22*, 1801–1808.
39. Nes, W.D., Norton, R.A., and Benson, M. (1992) *Phytochemistry 31*, 805–811.
40. Nes, W.D., Benson, M., Lundin, R.E., and Le, P.H. (1988) *Proc. Natl. Acad. Sci. USA 85*, 5759–5763.
41. Xu, S., Norton, R.A., Crumley, F.G., and Nes, W.D. (1988) *J. Chromatogr. 452*, 377–398.
42. Nes, W.D., and Le, P.H. (1990) *Biochim. Biophys. Acta 1042*, 119–125.
43. Nes, W.D., Janssen, G.G., Crumley, F.G., Kalinowska, M., and Akihisa, T. (1993) *Arch. Biochem. Biophys. 300*, 724–733.
44. Chitwood, D.J., and Patterson, G.W. (1991) *J. Liq. Chromatogr. 14*, 151–163.

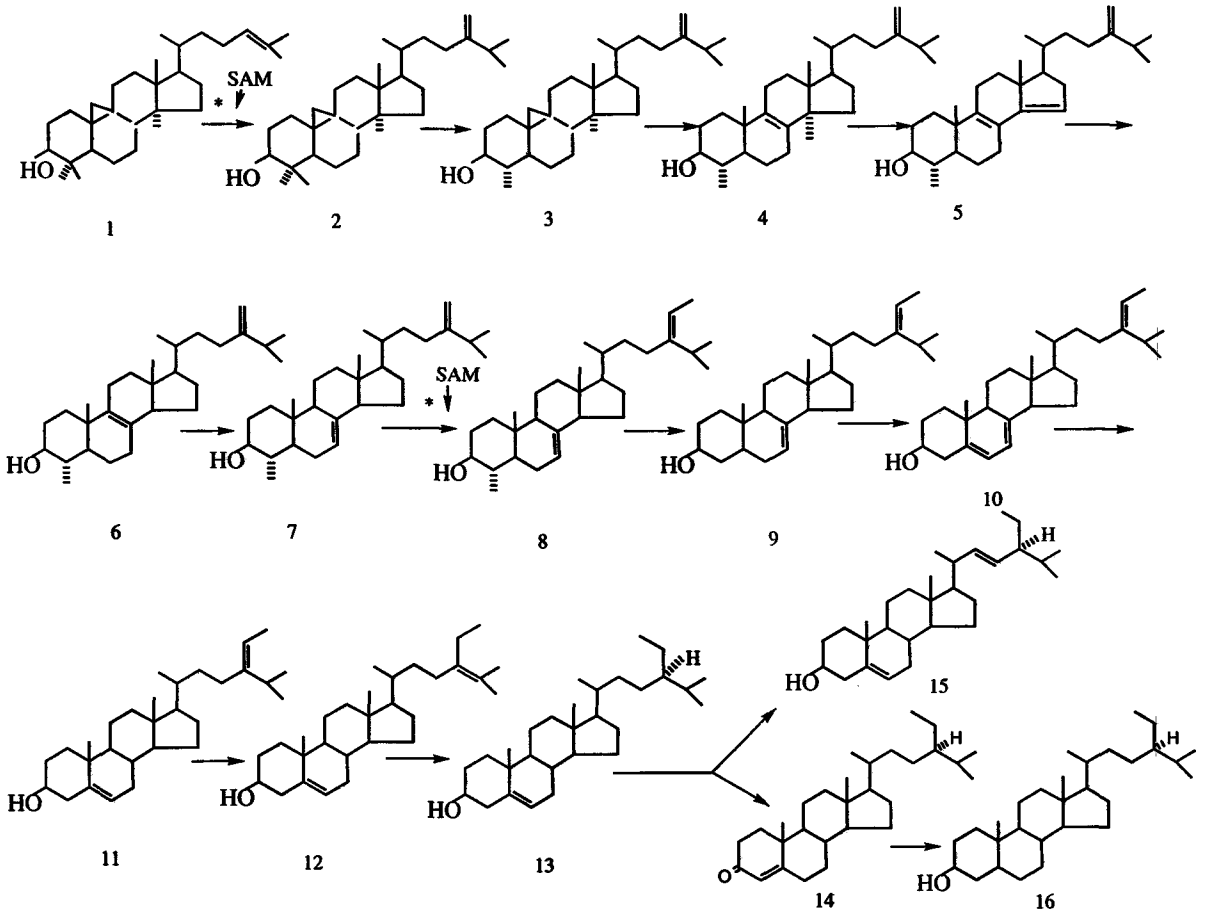


FIG. 15. Hypothetical kinetically favored pathway of phytosterol transformation in corn blades. Stars represent branch points which may give rise to alternate 24-alkyl-sterol products and hence to alternate sterol transformation pathways that may produce multiple 24-alkyl-sterol end products, e.g., not shown are 24-methyl-sterols. SAM, *S*-adenosyl-L-methionine.

45. Nes, W.R. (1985) *Methods Enzymol. 111*, 3–37.
46. Uomori, A., Nakagawa, Y., Yoshimatsu, S., Seo, S., Sankawa, U., and Takeda, K. (1992) *Phytochemistry 31*, 1569–1572.
47. Nes, W.D., Norton, R.A., Crumley, F.G., Madigan, S.J., and Katz, E.R. (1990) *Proc. Natl. Acad. Sci. USA 87*, 7565–7569.
48. Lakshmann, M.R., and Veech, L.R. (1977) *J. Biol. Chem. 252*, 4667–4673.
49. Goad, L.J. (1983) *Biochem. Soc. Trans. 23*, 548–552.
50. Seo, S., Uomori, A., Yoshimura, Y., Seto, H., Ebizuka, Y., Noguchi, H., Sankawa, U., and Takeda, K. (1990) *J. Chem. Soc. Perkin Trans I*, 105–108.
51. Wagschal, K., Savage, T.J., and Croteau, R. (1991)*Tetrahedron 47*, 5933–5944.
52. Goad, L.J., Knapp, F.F., Lenton, J.R., and Goodwin, T.W. (1972) *Biochem. J. 129*, 219–222.
53. Le, P.H., and Nes, W.D. (1986) *Chem. Phys. Lipids 40*, 57–69.
54. Hartmann, M., and Benveniste, P. (1987) *Methods Enzymol. 148*, 632–650.
55. Seitz, L.M. (1989) *J. Agric. Food Chem. 37*, 662–667.
56. Nes, W.D., Janssen, G.G., and Bergenstrahle, A. (1991) *J. Biol. Chem. 266*, 15202–15212.
57. Janssen, G.G., and Nes, W.D. (1992) *J. Biol. Chem. 267*, 25856–25863.

[Received August 31, 1994, and in revised form January 5, 1995; Revision accepted January 18, 1995]

Cloning of the Late Genes in the Ergosterol Biosynthetic Pathway of *Saccharomyces cerevisiae*—A Review[1]

N.D. Lees[a,*], B. Skaggs[a], D.R. Kirsch[b] and M. Bard[a]

[a]Department of Biology, Indiana University–Purdue University at Indianapolis, Indianapolis, Indiana 46202-5132 and [b]Agricultural Research Division, American Cyanamid, Princeton, New Jersey 08543-0400

ABSTRACT: Research on the ergosterol biosynthetic pathway in fungi has focused on the identification of the specific sterol structure required for normal membrane structure and function and for completion of the cell cycle. The pathway and its end product are also the targets for a number of antifungal drugs. Identification of essential steps in ergo-sterol biosynthesis could provide new targets for the development of novel therapeutic agents. Nine of the eleven genes in the portion of the pathway committed exclusively to ergosterol biosynthesis have been cloned, and their essentiality for aerobic growth has been determined. The first three genes, *ERG9* (squalene synthase), *ERG1* (squalene epoxidase), and *ERG7* (lanosterol synthase), have been cloned and found to be essential for aerobic viability since their absence would result in the cell being unable to synthesize a sterol molecule. The remaining eight genes encode enzymes which metabolize the first sterol, lanosterol, to ultimately form ergosterol. The two earliest genes, *ERG11* (lanosterol demethylase) and *ERG24* (C-14 reductase), have been cloned and found to be essential for aerobic growth but are suppressed by mutations in the C-5 desaturase (*ERG3*) gene and *fen1* and *fen2* mutations, respectively. The remaining cloned genes, *ERG6* (C-24 methylase), *ERG2* (D8Æ7 isomerase), *ERG3* (C-5 desaturase), and *ERG4* (C-24(28) reductase), have been found to be nonessential. The remaining genes not yet cloned are the C-4 demethylase and the C-22 desaturase *(ERG5).*
Lipids 30, 221–226 (1995).

Sterol biosynthesis in eucaryotic cells has been intensely studied for decades. The biosynthetic pathways of animal, plant, and fungal sterols are now well-defined, and the regulation of cholesterol biosynthesis in animal cells has been well documented (1). Much of the interest in the cholesterol pathway has resulted from the association of poorly regulated cholesterol synthesis and cardiovascular disease.

Research on the fungal sterol pathway which produces the C_{28} sterol, ergosterol, as its end product has followed two major lines of investigation. The first of these is directed to answer fundamental questions concerning the role or roles of sterol in membrane structure and function. Using a variety of systems, sterols have been shown to play critical roles in maintaining appropriate membrane fluidity (2,3), regulating membrane permeability (4,5), influencing the activity of membrane-bound enzymes (6), and altering cell growth rate (7). Beyond these membrane-related functions, sterols have been implicated in providing an essential function in the cell cycle. In this capacity, a sterol of specific structural features is required at hormonal levels (10 nM) to allow cell cycle completion. This phenomenon has been demonstrated in yeast (8) and animal cells (9,10). The elucidation of this sterol function as well as the sterol molecule responsible have been subjects of recent research. The second focus of fungal sterol research is driven by the need to develop new antifungal agents for use in medicine and agriculture. There has been an alarming recent rise in the number of human fungal infections (11) resulting from several factors that include both increases in the incidence of immunosuppressive disease and increases in immunosuppression resulting from technological advances in areas of science and medicine that have modified clinical practices.

As eucaryotes, fungal pathogens possess few potential therapeutic targets that would leave the host unaffected. The rise in microbial resistance to currently available therapeutic agents has been cited in publications varying from basic scientific journals to the lay press (12,13). Although the major emphasis of many of these articles is bacterial resistance, all officially approved antifungal agents have major deficiencies that limit their effective use. Of additional consequence here is the fact that some of the best fungal antibiotics are fungistatic rather than fungicidal. Coupled with immunosuppressive disease and medical advances in chemotherapy and transplantation, all of which result in or require immunosuppression, a fungistatic compound merely retards the pathogen which must then be controlled by a nonfunctional immune response. Human immunodeficiency virus infection is a particularly important area in which currently available antifungals have

[1]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids", Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at Department of Biology, Indiana University–Purdue University at Indianapolis, 723 West Michigan St., Indianapolis, IN 46202-5132.

Abbreviations: PCR, polymerase chain reaction; SBI, sterol biosynthesis inhibitor.

Copyright © 1995 by AOCS Press

been of limited efficacy (14,15). Thus, the identification of essential or required steps in sterol synthesis might provide ideal targets for new drugs that could have improved antifungal activity.

The major targets for antifungal compounds include ergosterol and its biosynthesis. There are a number of drugs that specifically block ergosterol biosynthesis with little effect on host cholesterol synthesis. Specificity is based upon differential enzyme susceptibility or differences in cell permeability. In many cases, adverse side effects result from inhibition of host enzymes. The allylamines inhibit squalene epoxidase (16), an enzyme appearing in the pathway just before the synthesis of the first sterol molecule (Fig. 1). These compounds are restricted to primarily topical applications and show little activity against pathogens that produce life-threatening systemic infections. The morpholines (17) are a group of sterol biosynthesis inhibitors (SBI) which block two separate steps in sterol synthesis, C-14 sterol reduction (*ERG24*) and sterol Δ8→7 isomerization (*ERG2*). This group is used primarily in agricultural applications although amorolfine is under investigation for human topical use (18). The azoles (imidazole and triazoles) are the most widely employed SBI (19) and are used in both topical and systemic situations. These compounds inhibit the C-14 demethylation of lanosterol (*ERG11* gene product) resulting in the formation of C-14 methylsterols. As a substitute for ergosterol, these sterols produce membrane effects leading to reduced growth (20), decreased membrane fluidity (3), and reduced ability to form invasive mycelia (20), an important feature in fungal pathogenesis. The polyene group (21) of antifungals interacts selectively with membrane ergosterol and introduce channels through which essential cell constituents escape, resulting in cell death. Due to their hydrophobicity and metabolism, some polyenes, such as nystatin, are restricted to topical application. Others, such as amphotericin B, can be used systemically but produce serious side effects.

Although not recognized as a major pathogen (22), the yeast *Saccharomyces cerevisiae* has been used as a model system for the elucidation of the ergosterol pathway, and it has been possible in this organism to determine the essentiality of the genes responsible for some of the enzymatic steps. Yeast has the advantage of a defined life cycle and sexual reproduction, making haploid existence and genetic crosses routine. Progress using pathogenic fungi, such as *Candida albicans*, has been considerably slower and largely dependent on procedures and information derived from work with *S. cerevisiae*.

The review will focus on the latter part of the pathway, beginning with the synthesis of squalene, the precursor of lanosterol, which is the first sterol intermediate in the pathway (Fig. 1). Steps prior to squalene formation are important for pathway regulation, and early pathway intermediates are me-

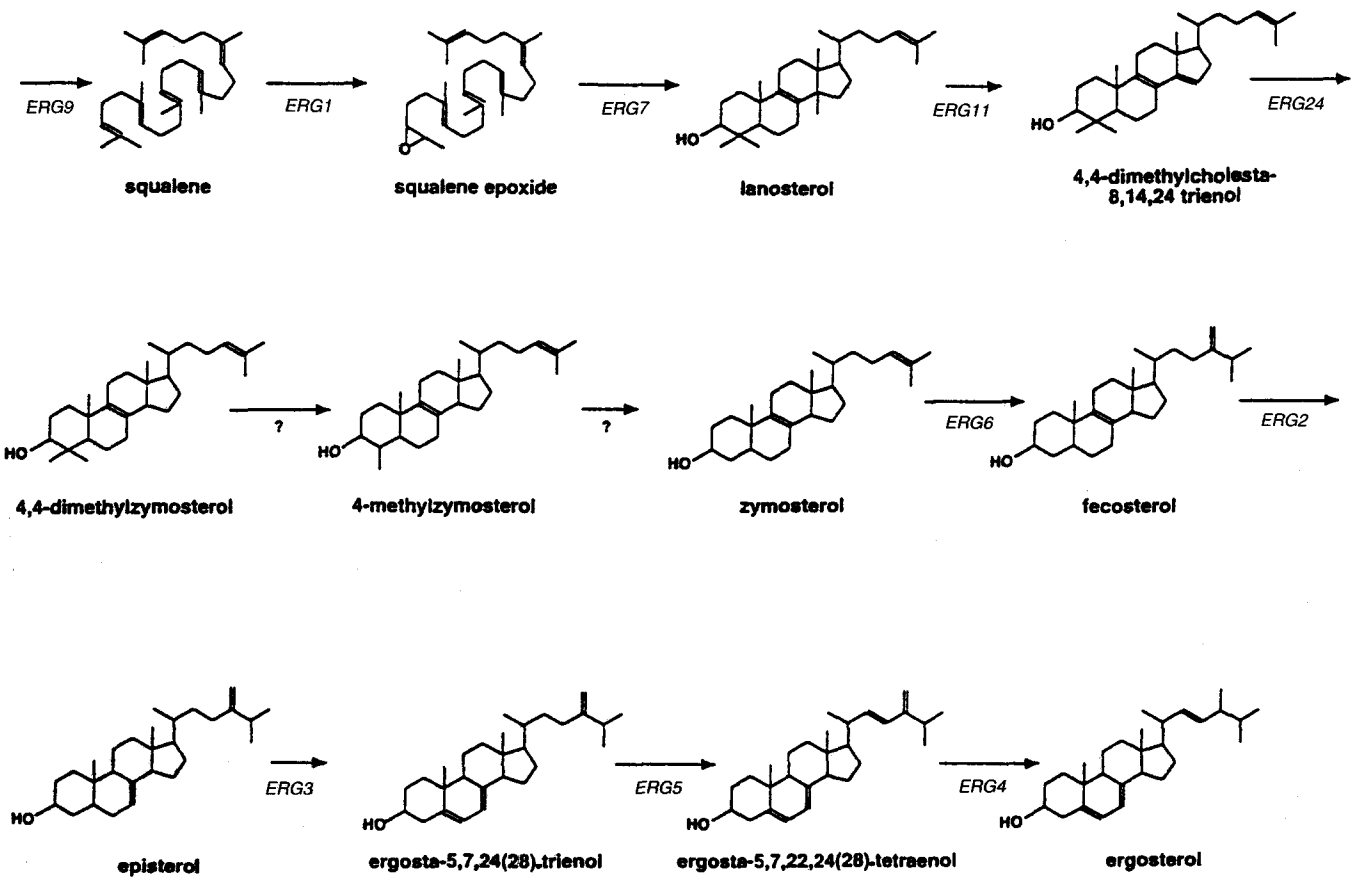


FIG. 1. The sterol biosynthetic pathway in *Saccharomyces cerevisiae* (adapted from Ref. 57).

tabolized to produce other essential cellular components (1). The genes responsible for coding nine of the eleven enzymes in the squalene to ergosterol part of the pathway have been cloned, and the essentiality of each for aerobic growth has been determined. For biosynthetic steps from lanosterol to ergosterol, essentiality was determined by gene disruption, followed by allelic replacement and growth under aerobic conditions. Blocks in the steps from squalene formation to lanosterol synthesis are essential because they do not allow the formation of any sterol molecule. Table 1 lists the eleven reactions from lanosterol formation to ergosterol and, where known, the genes involved, their essentiality for aerobic growth, their chromosomal location and drugs that inhibit the gene products. The genes specifying the enzymes responsible for two of the eleven steps have not been cloned. These include the genes encoding the enzymes responsible for the removal of the two bulky C-4 methyl groups and for desaturation of the side chain at the C-22 position (*ERG5*).

ERG9

Squalene, the first sterol precursor in ergosterol biosynthesis, is formed from two farnesyl diphosphate molecules by the enzyme squalene synthetase (farnesyl diphosphate farnesyl transferase). The substrate of this reaction, farnesyl diphosphate, serves as a beginning compound in the synthesis of several other important molecules that have isoprenoid components. Thus, the action of the *ERG9* gene product represents the first reaction in the branch committed to sterol synthesis. Two laboratories have reported the cloning of the *ERG9* gene (23,24). In both studies, *erg9* sterol auxotrophs containing a marker allowing the aerobic uptake of sterol were transformed with a wild-type yeast genomic library. Transformants capable of aerobic growth without ergosterol supplementation were selected. Plasmids containing the *ERG9* gene resulted in squalene synthase activity in *erg9* cells as well as in *Escherichia coli*. Characterization of the insert DNA indicated an open reading frame coding for a 444 amino acid protein with a molecular mass of approximately 51,700. This enzyme is highly conserved between yeast and humans (25). The *ERG9* gene has been mapped to chromosome 8.

ERG1

The product of the *ERG1* gene is an oxygen-requiring enzyme that converts squalene to 2,3-oxidosqualene, the immediate precursor of lanosterol. This enzyme is sensitive to allylamine inhibition, and this characteristic has been exploited to clone *ERG1* (26). An allylamine-resistant mutant (based on enzyme resistance) of *S. cerevisiae* was used to construct a library that was employed to transform the wild-type parental strain. Transformants were then isolated based on resistance to the drug. The plasmid containing the *erg1* allele was analyzed and found to contain an open reading frame encoding a 496 amino acid protein. The *ERG1* gene has been localized to chromosome 15.

ERG7

The *ERG7* gene encoding 2,3-oxidosqualene cyclase or lanosterol synthase has been cloned from both *C. albicans* (27) and, more recently, *S. cerevisiae* (28). A similar gene encoding the plant enzyme, cycloartenol synthase, which performs a parallel step in plant sterol synthesis, has also been cloned from *Arabidopsis thaliana* (29). In *S. cerevisiae*, polymerase chain reaction (PCR) amplification of yeast genomic DNA was performed using oligonucleotide primers derived from the conserved amino acid sequences from the *Candida* and *Arabidopsis ERG7* gene products. The PCR product was then employed to probe both cDNA and genomic libraries, but no full-length sequences were identified, possibly because the sequence is unstable in *E. coli*. Ultimately, two overlapping clones were utilized to reconstruct the entire coding sequence. The gene comprises a 2196bp open reading frame corresponding to a 732 amino acid protein. As is the case with the other genes preceding the formation of a sterol molecule, *ERG7* is required for aerobic viability. The *Candida ERG7* gene (27) was cloned by using a wild-type *C. albicans* library

TABLE 1
Cloned Genes in the Ergosterol Biosynthetic Pathway

Gene	Enzyme encoded	Essential for viability	Known enzyme inhibitors	Chromosomal location
ERG9	Squalene synthase	Yes	Squalestatin	8
ERG1	Squalene epoxidase	Yes	Allylamines	15
ERG7	Squalene cyclase	Yes	U1866A	8
ERG11	Lanosterol demethylase	Yes[a]	Azoles	8
ERG24	Δ14 Reductase	Yes[b]	Morpholines	?
ERG6	C-24 Methyltransferase	No		13
ERG2	C-8 Isomerase	No	Morpholines	13
ERG3	C-5 Desaturase	No		12
ERG4	Δ24 Reductase	No		7

[a]Suppressed by *erg3*.
[b]Suppressed by *fen1* and *fen2*.

to complement an *erg7* mutant of *S. cerevisiae*. *ERG7* has been mapped to chromosome 8 in *S. cerevisiae*.

ERG11

The product of the *ERG11* gene is a cytochrome P-450 demethylase which is responsible for the removal of the methyl group from the C-14 position. Several aerobically viable *erg11* mutants have been reported (30,31), but all have been found to be leaky and were accompanied by a second mutation in the *ERG3* gene whose product introduces a double bond at the C-5 position (C-5 desaturase) later in the pathway. The essentiality of the *ERG11* gene was in question until it was cloned and characterized by Kalb *et al.* (32,33) and found to be essential for aerobic growth. The cloning was accomplished by transforming yeast with a yeast genomic library in a high copy number plasmid and screening transformants for resistance to the azole, ketoconazole, a specific inhibitor of the C-14 demethylase. Subsequent analysis indicated a protein of 530 amino acids. The gene encoding the demethylase has been mapped to chromosome 8.

The suppression of *ERG11* essentiality by *erg3* mutants remained a question. Recently, Bard *et al.* (34) have shown that double disruptions of *ERG11* and *ERG3* are aerobically viable, indicating that the removal of the C-14 methyl group is not an essential step. The suppression of the *erg11* phenotype by *erg3* is explained by the hypothesis of Watson *et al.* (35). Since the enzymes of the sterol pathway show a high level of substrate infidelity, enzymes subsequent to a blocked step will continue to catalyze the available sterol intermediates. In this case, C-14 methyl sterols continue down the pathway to the C-5 desaturase which attempts to desaturate the C-5 position but is unable to complete the process. The result of this aborted effort is the formation of an intermediate, 14α-methyl-ergosta-8,24(28)-diene-3β,6α-diol, a sterol proposed to disrupt membrane structure and function resulting in cell death. This diol has been identified in *erg11* mutants of *C. albicans* and is tolerated by this organism (36,37). This difference may be the result of subtle variabilities in membrane structure or may reflect alternative mechanisms for esterification of toxic sterols in the two species.

ERG24

The product of the C-14 demethylase reaction, 4,4-dimethylcholesta-8,14,24-trienol, is reduced at the C-14 position to form 4,4-dimethylcholesta-8,24-dienol by the action of the product of the *ERG24* gene. The C-14 reductase is one of the two sites inhibited by the morpholine antibiotics, and this characteristic was employed by one group to clone *ERG24* (38). Using a strategy similar to that employed in the cloning of *ERG11*, *S. cerevisiae* was transformed with a yeast genomic library in a high copy number plasmid, and resulting transformants were screened for resistance to fenpropidin, a morpholine. Resistance was confirmed to be due to the overproduction of the C-14 reductase. Subsequent gene disruption and allele replacement resulted in strains that were unable to grow in the presence of oxygen. To address the issue of whether nonviability under these conditions is due to the accumulation of toxic sterol intermediate (primarily ignosterol, ergosta-8,14-dienol) or the lack of a sterol possessing an essential structural feature, aerobic growth was attempted in a genetic background allowing for uptake of exogenous sterols on a medium containing ergosterol. Growth did occur, arguing that ignosterol did not block aerobic growth.

Simultaneously, a second research group cloned the C-14 reductase using a clever alternative strategy (39). This group selected for a fenproprimorph-resistant mutant that carried two recessive mutations, *fen1* and *fen2*. Both the wild-type and the resistant mutant produce ergosterol when the drug is absent and ignosterol when it is present. The presence of the *fen1* and *fen2* mutations allow survival of cells producing only ignosterol. Nystatin-resistant isolates of a strain carrying *fen1* and *fen2* were generated by mutagenesis and screened for the accumulation of ignosterol. One such isolate was obtained. The *ERG24* gene was cloned by transforming the nystatin-resistant, ignosterol-producing, *fen1, fen2* strain with a wild-type yeast genomic library and by selecting an ergosterol-producing, nystatin-sensitive isolate. The plasmid conferring the desired phenotype was characterized, and a 1314bp open reading frame encoding a 438 amino acid protein of a molecular mass of 50,612 was deduced. This gene has yet to be mapped.

A third research group has recently reported the cloning and characterization of the *ERG24* gene (40). The approach used was similar to that employed by Marcireau *et al.* (38). Sequence analysis confirmed the conclusions of the previous reports. In addition, this study reported sequence similarities among *ERG24*, the *sts1*$^+$ gene of *Schizosaccharomyces pombe* (41) and the *YGL022* gene of *S. cerevisiae* (42). The latter two genes and their relationship to the ergosterol pathway are discussed in the *ERG4* section of this review.

ERG6

The product of the *ERG6* gene methylates the C-24 position of zymosterol to produce the 28 carbon sterol structure common to most fungi. C-24 methylation was considered to be an essential step in the synthesis of functional sterol (43,44). However, since only hormonal amounts of "essential" sterol were required, absolute proof of this hypothesis was difficult. The *ERG6* gene was ultimately cloned by complementation using an *erg6* mutant (45). Cloning using the *erg6* strain presented technical difficulties since cell membrane function was significantly affected by the presence of unmethylated sterol, resulting in low transformation rates and diminished mating efficiencies. Significant protocol alterations were required to successfully transform *erg6* with a yeast genomic library and select for clones that were insensitive to cycloheximide (a permeability defect in *erg6* results in increased sensitivity to this drug). Restoration of wild-type cycloheximide resistance was an effective initial screen for *erg6* complementation.

Transformants were then assayed for ergosterol production and nystatin sensitivity. Two deletion-substitutions of the *ERG6* gene were constructed on a plasmid used to replace the wild-type *ERG6* gene by transformation in which the *LEU2* gene inserted into the *ERG6* gene provided a selectable marker. These disrupted strains were viable. A subsequent report (46) has indicated that gene *SED6*, which suppressed a mutant form of *ERD2*, a gene involved in protein retrieval from endoplasmic reticulum, is identical to the *ERG6* gene. The *ERG6* gene has been localized on chromosome 13.

ERG2

The Δ8→7 isomerase, the product of the *ERG2* gene, is one of the targets of the morpholine antibiotics (47). Based on the efficacy of these drugs, it was suggested that this step may be essential. Using the same protocol as was used with *ERG6*, *erg2* mutants were transformed with a yeast genomic library and screened for cycloheximide resistance (48). Sequence analysis of functional plasmid inserts conferring ergosterol synthesis indicated an open reading frame encoding a 222 amino acid protein. Disruption of the coding sequence did not affect viability, indicating the gene is not essential (49). The *ERG2* gene has also been mapped to chromosome 13.

ERG3

The C-5 desaturase, the product of the *ERG3* gene, converts episterol [ergosta-7,24(28)-dienol] to ergosta-5,7,24(8)-trienol. Based on feeding experiments, Parks and co-workers (50–52) concluded that only sterols containing a C-5 unsaturation or the capability of undergoing this alteration could provide the essential sterol function. The *ERG3* gene was cloned (53) using a protocol for transformation, selection and verification identical to that employed for *ERG6* (45) and *ERG2* (48). Sequencing of the *ERG3* gene indicated a 365 amino acid protein. The essentiality of *ERG3* was determined by deletion and substitution, and strains carrying the allele containing the deletion and disruption are viable. The *ERG3* gene is located on yeast chromosome 12.

ERG4

The *ERG4* gene provides the enzyme which reduces the double bond at C-24(28), the final step in ergosterol biosynthesis. Direct cloning of this gene has not been possible using the protocols employed in cloning the genes operating earlier in the pathway. The sterols accumulated in *erg4* mutants are similar in structure to ergosterol, and the effects of sterol substitution are insufficient to distinguish mutant and wild-type, based on permeability or antibiotic sensitivity differences. Recently, the sequence for *ERG24* was shown (40) to have similarity to the sequences reported for the *sts1*$^+$ gene of *S. pombe* (41), the *YGL022* gene of *S. cerevisiae* (42) and the chicken nuclear lamin B receptor gene (54). Based on the accumulation of C-24(28) sterols, both *sts1*$^+$ and *YGL022* were determined to contain defects in the C-24(28) reductase (*ERG4*). In addition, a disruption of *YGL022* was shown to be allelic to an *erg4* mutant. The authors thus concluded that *YGL022* is *ERG4* and that the reductase is not essential for viability. Figure 2 shows the amino acid sequences of the *ERG24* and *ERG4* gene products. *ERG4* has been localized to chromosome 7.

THE REMAINING GENES

The remaining two steps not yet cloned in the ergosterol biosynthetic include the *ERG5* gene and the C-4 demethylase gene. The latter gene is interesting since no mutants have been detected using the standard polyene resistance selection procedure. This might mean that the inability to demethylate may result in the accumulation of sterol intermediates that are unacceptable for functional inclusion in the membrane or that the resulting sterols may not possess the essential structural features to provide the essential cell cycle function. The process of C-4 demethylation is complex and involves three separate enzymes—an oxidase, a decarboxylase, and a 3-keto reductase. Little work has been done on the yeast enzymes although the counterpart reactions in the cholesterol pathway have been described (55,56).

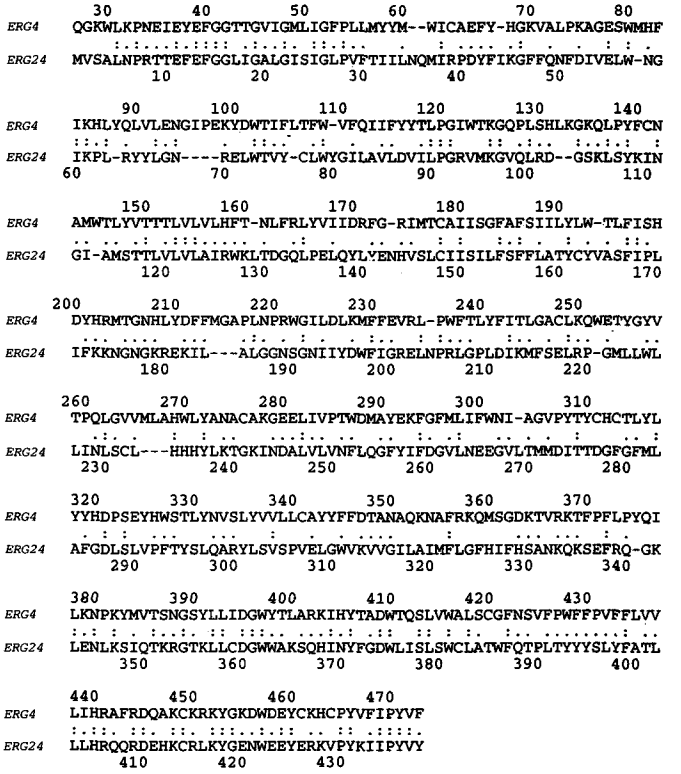


FIG. 2. The amino acid sequences of the *ERG4* and *ERG24* genes of *Saccharomyces cerevisiae*. Single dots represent amino acid similarity and double dots represent amino acid identity.

ACKNOWLEDGMENTS

Various aspects of the research summarized in the review were supported in part by an NIH grant IR15GM45959-01, a grant from Eli Lilly & Co. and an American Cyanamid contract, all to M.B. We thank Mark Goebl for providing the figure containing the sequences for *ERG24* and *ERG4*.

REFERENCES

1. Goldstein, J.L., and Brown, M.S. (1990) *Nature 243*, 425–430.
2. Lees, N.D., Bard, M., Kemple, M.D., Haak, R.A., and Woods, R.A. (1979) *Biochim. Biophys. Acta 553*, 469–475.
3. Lees, N.D., Kleinhans, F.W., Broughton, M.C., Pennington, P.A., Ricker, V.A., and Bard, M. (1989) *Steroids 53*, 567–578.
4. Bard, M., Lees, N.D., Burrows, L.S., and Kleinhans, F.W. (1978) *J. Bacteriol. 135*, 1146–1148.
5. Kleinhans, F.W., Lees, N.D., Bard, M., Haak, R.A., and Woods, R.A. (1979) *Chem. Phys. Lipids 23*, 143–154.
6. Rottem, S., Yaskoav, J., Neeman, Z., and Razin, S. (1973) *Biochim. Biophys. Acta 323*, 495–508.
7. Lees, N.D., Lofton, S.L., Woods, R.A., and Bard, M. (1980) *J. Gen. Microbiol. 118*, 209–214.
8. Dahl, C., Biemann, H.P., and Dahl, J. (1987) *Proc. Natl. Acad. Sci. USA 84*, 4012–4016.
9. Quesney-Huneeus, V., Wiley, M.H., and Siperstein, M.D. (1979) *Proc. Natl. Acad. Sci. USA 76*, 5056–5060.
10. Quesney-Huneeus, V., Galick, H.A., Siperstein, M.D., Erikson, S.K., Spencer, J.A., and Nelson, J.A. (1983) *J. Biol. Chem. 258*, 378–385.
11. Georgopapadakou, N.H., and Walsh, T.J. (1994) *Science 264*, 371–373.
12. Travis, J. (1994) *Science 264*, 360–362.
13. Davies, J. (1994) *Science 264*, 375–382.
14. Klein, R.S., Harris, C.A., Small, C.B., Moll, B., Lesser, M., and Friedland, G.H. (1994) *New Engl. J. Med. 311*, 554–556.
15. Beyer, J., Schwartz, S., Heinemann, V., and Siegert, W. (1994) *Antimicrob. Agents Chemother. 38*, 911–917.
16. Jolidon, S., Polack, A.M., Guerry, P., and Hartman, P.G. (1989) *Biochem. Soc. Trans. 18*, 47–49.
17. Marcireau, C., Guilloton, M., and Karst, F. (1990) *Antimicrob. Agents Chemother. 34*, 989–993.
18. Hartman, G., and Polak, A. (1993) *Clin. Dermatol. 7*, 27–36.
19. Vanden Bossche, H., Willemsens, G., Cools, W., Cornelissen, F., Lauwers, W.F., and Van Cutsem, J. (1980) *Antimicrob. Agents Chemother. 17*, 922–928.
20. Lees, N.D., Broughton, M.C., Sanglard, P., and Bard, M. (1990) *Antimicrob. Agents Chemother. 34*, 831–836.
21. Kerridge, D. (1975) *Postgrad. Med. J. 55*, 653–656.
22. McCasker, J.H., Clemons, K.V., Stevens, D.A., and Davis, R.W. (1994) *Genetics 136*, 1261–1269.
23. Fegueur, M., Richard, L., Charles, N.D., and Karst, F. (1991) *Curr. Genet. 20*, 365–372.
24. Jennings, S.M., Tsay, Y.H., Fisch, T.M., and Robinson, G.W. (1991) *Proc. Natl. Acad. Sci. USA 88*, 6038–6042.
25. Robinson, G.W., Tsay, Y.H., Kienzle, B.K., Smith-Monroy, C.A., and Bishop, R.W. (1993) *Mol. Cell Biol. 13*, 2706–2717.
26. Jandrositz, A., Turnowsky, F., and Hogenauer, G. (1991) *Gene 107*, 155–160.
27. Kelly, R., Miller, S.M., Lai, M.H., and Kirsch, D.R. (1990) *Gene 87*, 177–183.
28. Covey, E.J., Matsuda, S.P.T., and Bartel, B. (1994) *Proc. Natl. Acad. Sci. USA 91*, 2211–2215.
29. Covey, E.S., Matsuda, S.P.T., and Bartel, B. (1993) *Proc. Natl. Acad. Sci. USA 90*, 11628–11632.
30. Trocha, P.J., Jasne, S.J., and Sprinson, D.B. (1977) *Biochemistry 16*, 4721–4726.
31. Taylor, F.R., Rodriquez, F.J., and Parks, L.W. (1983) *J. Bacteriol. 156*, 64–68.
32. Kalb, V.F., Loper, J.C., Dey, C.R., Woods, C.W., and Sutter, T.R. (1986) *Gene 45*, 237–245.
33. Kalb, V.F., Woods, C.W., Turi, T.G., Dey, C.R., Sutter, T.R., and Loper, J.C. (1987) *DNA 6*, 529–537.
34. Bard, M., Lees, N.D., Turi, T., Craft, D., Cofrin, L., Barbuch, R., Koegel, C., and Loper, J.C. (1993) *Lipids 28*, 963–967.
35. Watson, P.F., Rose, M.E., Ellis, S.W., England, H., and Kelly, S.L. (1989) *Biochem. Biophys. Res. Commun. 164*, 1170–1175.
36. Shimokawa, O., Kato, Y., and Nakayama, H. (1986) *J. Med. Vet. Mycol. 24*, 327–336.
37. Shimokawa, O., Kato, Y., Kawano, K., and Nakayama, H. (1989) *Biochim. Biophys. Acta 1003*, 15–19.
38. Marcireau, C., Guyonnet, D., and Karst, F. (1992) *Curr. Genet. 22*, 267–272.
39. Lorenz, R.T., and Parks, L.W. (1992) *DNA Cell Biol. 11*, 685–692.
40. Lai, M.H., Bard, M., Pierson, C.A., Alexander, J.F., Goebl, M., Carter, G.T., and Kirsch, D.R. (1994) *Gene 140*, 41–49.
41. Shimanuki, M., Goebl, M., Yanagida, M., and Toda, T. (1992) *Mol. Biol. Cell 3*, 263–273.
42. Chen, W., Capieaux, E., Balzi, E., and Goffeau, A. (1991) *Yeast 7*, 305–308.
43. Pinto, W.J., and Nes, W.R. (1983) *J. Biol. Chem. 258*, 4472–4476.
44. Pinto, W.J., Lozano, R., Sekula, B.C., and Nes, W.R. (1983) *Biochem. Biophys. Res. Commun. 112*, 47–54.
45. Gaber, R.F., Copple, D.M., Kennedy, B.K., Vidal, M., and Bard, M. (1989) *Mol. Cell Biol. 9*, 3447–3456.
46. Hardwick, K.C., and Pelham, H.R.B. (1994) *Yeast 10*, 265–269.
47. Baloch, R.I., Mercer, E.I., Wiggins, T.E., and Baldwin, B.C. (1984) *Phytochemistry 23*, 2219–2226.
48. Ashman, W.H., Barbuch, R.J., Ulbright, C.E., Jarrett, H.W., and Bard, M. (1991) *Lipids 26*, 628–632.
49. Arthington, B.A., Hoskins, J., Skatrud, P.L., and Bard, M. (1991) *Gene 107*, 173–174.
50. Rodriguez, R.J., Low, C., Bottema, C.D.K., and Parks, L.W. (1985) *Biochim. Biophys. Acta 387*, 336–343.
51. Rodriguez, R.J., and Parks, L.W. (1983) *Arch. Biochem. Biophys. 225*, 861–871.
52. Lorenz, R.T., Casey, W.M., and Parks, L.W. (1989) *J. Bacteriol. 171*, 6169–6173.
53. Arthington, B.A., Bennett, L.G., Skatrud, P.L., Guyra, C.J., Barbuch, R.J., Ulbright, C.E., and Bard, M. (1991) *Gene 102*, 39–44.
54. Worman, H.J., Evans, C.D., and Blobel, G. (1990) *J. Cell Biol. 111*, 1535–1542.
55. Fukishima, H., Grimstead, G.F., and Gaylor, J.F. (1981) *J. Biol. Chem. 256*, 4822–4826.
56. Faust, J.R., Trzaskos, J.M., and Gaylor, J.F. (1988) in *Biology of Cholesterol* (Yeagle, P.L., ed.) CRC Press, Boca Raton.
57. Paltauf, F., Kohlwein, S.D., and Henry, S.A. (1992) in *The Molecular and Cellular Biology of the Yeast Saccharomyces* (Jones, E.W., Pringle, J.R., and Broach, J.R., eds.) Vol. 2, Cold Spring Harbor Laboratory Press, New York.

[Received June 10, 1994, and in revised form December 19, 1994; Revision accepted December 21, 1994]

Biochemical and Physiological Effects of Sterol Alterations in Yeast—A Review[1]

Leo W. Parks*, Steven J. Smith and James H. Crowley

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7915

ABSTRACT: Considerable progress has been made in the selection and characterization of mutants that are defective in the synthesis of ergosterol in the yeast, *Saccharomyces cerevisiae*. Mutations in nearly every step of the yeast sterol biosynthetic pathway have been induced and selected. These mutants have been used to elucidate the sequential order of steps in sterol synthesis, to study the mode of action of antifungal agents and to determine the method of resistance of some pathogenic fungi, and to answer questions on the role of sterols in general cell biology. Physiological examination of ergosterol null mutants, lacking all biochemical activity attributed to the particular gene, supports a role for ergosterol in a number of critical functions in the organism. Among the physiological functions attributed to ergosterol are sparking and bulking requirements, involvement in amino acid and pyrimidine transport, resistance to antifungal agents and certain cations, and a requirement for respiratory activity. Those genetic null alleles discussed in this review are *erg24*, lacking the ability to reduce the Δ^{14} double bond; *erg6*, unable to methylate C-24; and *erg3*, defective in the C-5 desaturase. The different biochemical activities that are disrupted in the ergosterol mutants support a role for ergosterol in a number of critical functions in yeast.

Lipids 30, 227–230 (1995).

Considerable research is aimed toward understanding the roles of sterols in the physiology and biochemistry of the cell. Yeast is ideally suited for this work because of its ease of culture, well-defined genetic and molecular biological procedures, and the wealth of biochemical data that is available on these organisms. Ten years ago, experiments were performed that indicated that there were multiple roles for sterols in yeast (1). Prior to that, it was generally assumed that sterols in fungi merely served passive roles in their constituent membranes to buffer the membrane's physical properties in response to environmental changes. Working with the assumptions that if different functions existed they would likely have different quantitative and qualitative requirement for the sterols and that ergosterol served as a "consensus" sterol, it was established that at least four growth-dependent functions for sterols must exist. The inability to define the biochemical and physiological components of these functions has been a continuing challenge in this field. Here the status of the studies on sterol functions will be reviewed, particularly with regard to developments with the *ERG6*, *ERG24*, and *ERG3* structural genes.

ERG6

ERG6 is the structural gene for the sterol methyltransferase. In yeast, zymosterol is the preferred substrate with the formation of fecosterol at the expense of *S*-adenosylmethionine. In respirationally adapting cells we have found small amounts of 4α-methyl fecosterol in our cultures (2). The *erg6* mutants have been relatively easy to obtain and, in fact, are the most abundant class of ergosterol mutants that have been isolated. However, these were also a source of enormous frustration to us in our early experiments. Working with a haploid strain that had a tryptophan marker for genetic purposes, a wide variety of procedures were attempted to select for nystatin-resistant mutants that were defective in sterol methyltransferase, but these attempts were without success. Many *erg3* mutants were isolated, blocked in the C-5 desaturase, which other researches found difficult to obtain. It is clear now why such difficulties were experienced thanks to the work of Gaber *et al.* (3). These authors found that mutants defective in sterol transmethylation had a sixfold reduction in tryptophan uptake. Thus, *erg6* mutants could not be selected in a tryptophan auxotroph because of their extremely poor growth. Impaired tryptophan uptake also contributed to the incorrect mapping assignment of *ERG6* being linked to *TRP1* (4). Since tryptophan is transported by two permease systems, each of relatively low specificity (5,6), the absence of methylated sterols may have a more general effect than altering one of the tryptophan uptake mechanisms. These results explain in part the discrepancies in results obtained by different laboratories where different genetic constructions are used.

Additional transport defects in *erg6* mutants are suggested by recent work from Trumbly's laboratory (7). There the

[1]Based on a paper presented at the Symposium on "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at Department of Microbiology, North Carolina State University, Box 7615, Raleigh, NC 27695-7615.

Abbreviations: ER, endoplasmic reticulum; Erd2p, structural protein encoded by the ERD2 gene; Erg3p, structural protein encoded by the ERG3 gene.

Copyright © 1995 by AOCS Press

ERG6 structural gene was inadvertently selected in screenings for revertants to mutants that had hypersensitivity to certain cations, especially Li^+ and Na^+. It is now known that a gene originally identified as *LISI* is in fact *ERG6*. Previous work from Bard's laboratory (8) suggested that sterol mutants had enhanced sensitivity to cations.

Brefeldin A, which disassembles the Golgi structures and inhibits protein movement to the plasma membrane, has been used extensively in mammalian cell cultures. However, wild-type yeast are insensitive to it. The *erg6* mutants are sensitive to Brefeldin A and have been used to study the secretory process (9–11).

The *erg6* mutants also have decreased mating frequency (3). This reduction in zygote formation is consistent with our observations that sterols other than ergosterol reduce mating frequency (12). With several sterols other than ergosterol, we found that mated pairs became adherent but failed to undergo cytoplasmic fusion.

A most intriguing effect of *ERG6* has been found in mutants that have a defect in the secretory pathway. The trafficking of newly synthesized proteins that are destined for the vacuole, plasma membrane, or secretion in eukaryotic cells are sequentially passed through a series of compartments. Specifically targeted transport vesicles mediate the movement of the proteins with high precision and efficiency between the compartments. Some of the components of this secretory pathway have been defined genetically and biochemically (13). Newly synthesized proteins that are destined for the secretory pathway are inserted initially into the endoplasmic reticulum (ER). Subsequently they are transported to the Golgi and then partitioned to their appropriate destination. In the vesicularly mediated transport to the Golgi, some luminal proteins of the ER enter the transport vesicle, that buds from the ER, and are moved along with proteins that are destined for functions beyond the ER. These ER luminal proteins are returned to the ER following modification by Golgi specific enzymes (14). *ERD2* is a structural gene in yeast that encodes a receptor responsible for the retrieval of the ER proteins from the Golgi (15); mutants lacking this gene are unable to divide. A number of suppressors of *erd2*Δ strains have been isolated. One of these, *SED6*, is a weak multicopy suppressor and is effective when introduced into *erd2*Δ strains. Recently (16), following sequencing of *SED6*, it was found to be identical to *ERG6*. The role of the *ERG6* as a methyltransferase or some other function in permitting the replication of *erd2*Δ cells has not been determined.

Cells that are depleted of the *ERD2*-designated protein (Erd2p) accumulate large amounts of membranes that are unlike the transport vesicles, ER- or Golgi-like structures that are formed in other secretory-restricted strains (13). It is interesting that such Erd2p-depleted cells also abundantly accumulate lipid droplets. We have shown previously (4) that the cellular fraction most enriched with the sterol methyltransferase is the microdroplets of lipids, known as the floating lipid layer, that accumulate at the top of gradients when cells are fractionated (17).

Although the ERG6-designated protein, Erg6p, or its transmethylated sterol products appear to be involved in a variety of cellular activities, mutants lacking Erg6p are viable aerobically. Based on conclusions obtained by Atkinson (18), the transmethylation of zymosterol by *S*-adenosylmethionine is found to be very costly to the cell, occurring at an overall metabolic expense of from 12 to 14 ATP equivalents (19). It is highly unlikely that such an expensive reaction would be retained in the evolution of the organism, unless it offered a substantial selection advantage to organisms having a functional ERG6.

ERG24

The biosynthetic pathways for ergosterol and cholesterol include a $\Delta^{8,14}$-sterol intermediate as a consequence of the demethylation of lanosterol (20,21). Generally, the Δ^{14}-bond is reduced by the enzyme Δ^{14}-sterol reductase (22), forming 4,4-dimethylcholesta-8,14-dienol before the removal of the C-4 methyl groups or modifications of the sterol's side chain. We observed growth inhibition and the accumulation of ignosterol (ergosta-8,14-dien-3ß-ol) when yeast cells were treated with 15-azasterol (15-aza-24-methylene-D-homocholestadien-3ß-ol) (23,24). It is interesting that yeast cells growing on respiratory substrates were much more sensitive to 15-azasterol than cells growing on glucose (25). Fungal cells exposed to the morpholine antifungal agent, fenpropimorph, also accumulate ignosterol (26), and the antifungal activity has been attributed to the production of ignosterol (27,28). We have isolated mutants that are resistant to fenpropimorph (28), and we used them to clone and sequence the structural gene, *ERG24*, for the Δ^{14}-sterol reductase (29).

Fenpropimorph treatment results in a decrease in the level of membrane ergosterol and the accumulation of ignosterol and its derivatives. Consequences of the sterol alterations are most likely decreased capacity of the organisms to maintain normal membrane processes. It has been observed that the incorporation of 24-methylene-ignosterol into membranes results in increased membrane disorder and a concomitant decrease in the ability to accumulate glucose (30). We have found that fenpropimorph treatment of cells also affects directly the uptake of the pyrimidine bases, uracil and cytosine (31); however, the inhibition of uracil, and cytosine uptake differs dependent on the endogenous sterol produced (Fig. 1).

We have previously isolated mutants that are resistant to fenpropimorph (28), and these were used to clone the recessive *fen1* and *fen2* suppressors of *erg24*. We incorporated the *ura3* gene into our stocks so that we might use the *URA3*-containing YCp50 plasmid-based library for cloning *FEN1* and *FEN2*. We observed, however, that our strains became more resistant to the growth inhibitory effects of fenpropimorph, when made prototrophic for uracil by transformation. Cytosine, which is taken up by a different mechanism than is uracil (32,33), can rescue cells that have impaired uracil uptake as can high concentrations of uracil.

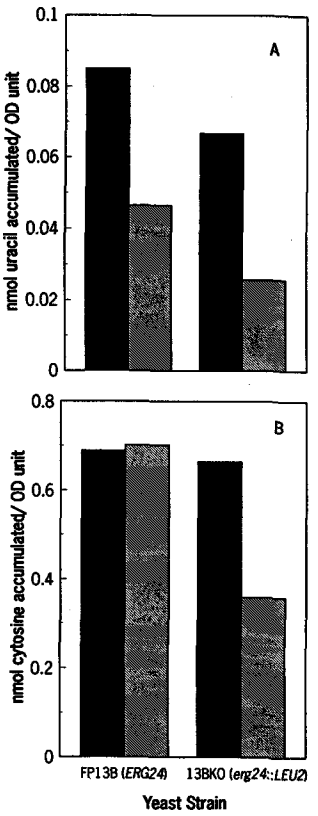


FIG. 1. Effect of fenpropimorph on the uptake of uracil and cytosine by *Saccharomyces cerevisiae*. Strains FP13B (*ERG24*) and 13BKO (*erg24::LEU2*), which synthesize ergosterol and ignosterol, respectively, were grown to exponential phase, filtered, resuspended in fresh medium with or without the addition of fenpropimorph, and incubated for 3 h. The uptake of radiolabeled uracil (A) and cytosine (B) was measured as described by Crowley et al. (31). Black bar, - Fenpropimorph; striped bar + Fenpropimorph.

ERG3

ERG3 is the structural gene for the sterol C-5 desaturase, which appears to function as a mixed-function oxidase (34). Working with strains of yeast that were defective in that desaturation, we found that cholestanol, lanosterol, or ergosta-7,22-dienol could satisfy the bulk of the required sterol but that a sterol with Δ^5-unsaturation was required for growth (35–37,38). When the *ERG3* gene was cloned and disrupted, Arthington *et al.* (39) found that their disrupted strains grew, despite their inability to synthesize Δ^5-sterols. The principal differences between the strains used by the Arthington group and our laboratory were that our strains had heme deficiencies which were included to permit the efficient uptake of exogenous sterols (40, 41). We found that insertionally activated mutants of *ERG3* did not grow without "sparking" levels of Δ^5-sterols in heme-deficient cells, and they did not grow on defined medium using respiratory substrates, such as glycerol and ethanol (38).

Although there is considerable circumstantial evidence in support of roles for sterols in the respiratory metabolism of cells, involvement of sterols at a specifically defined biochemical site has not been established. Toward that end, our laboratory is studying the effect of specific sterol deficiencies on respiratory development in strains of yeast. As a part of that study, we sought to determine which features of ergosterol might be essentially involved in synthesis or maintenance of respiratory competency. For that we took a genetic approach.

We reasoned that, as in our previous work (1), ergosterol could most likely satisfy all of the principal sterol functions in yeast. Yeast growing on ergosterol can grow respirationally or fermentatively. Yet, we assumed that if there were structural features of sterols that specifically did not permit respiration, we should be able to deduce them by differences between mutants that were selected on glucose as opposed to glycerol or ethanol. We undertook an extensive selection of mutants that were resistant to polyene antifungal agents on glucose or glycerol. A summary of the results is shown in Table 1. In total, 600 polyene-resistant mutants were selected, 300 on glucose and 300 on glycerol, and were characterized by sterol analysis. A wide variety of yeast mutant types were found on glucose. All of the mutants selected on glycerol as the carbon and energy source produced some Δ^5-sterols. Of the 300 selected, 297 have sterols that display the typical absorption spectrum of the $\Delta^{5,7}$-sterols. The remaining three have some $\Delta^{5,8}$-sterols. Most of the mutants produce the Δ^5-

TABLE 1
Isolation of *erg* Mutants on Fermentative and Respiratory Carbon Sources[a,b]

Media[c]	W303-1A	JC530-4b	x2180-1A
YPG + Nystatin	All mutants made some $\Delta^{5,7}$-sterol, often in addition to Δ^8-sterol.	All mutants made some $\Delta^{5,7}$-sterol, usually as $\Delta^{5,7,22,24(25)}$-sterol.	48 of 50 mutants made $\Delta^{5,7}$-sterol. Other two made $\Delta^{5,8,22}$-sterol or zymo.
YPG + Filipin	All mutants made some $\Delta^{5,7}$-sterol, often in addition to Δ^8-sterol.	All mutants made some $\Delta^{5,7}$-sterol, usually as $\Delta^{5,7,22,24(25)}$- or $\Delta^{24(28)}$-sterol.	49 of 50 mutants made $\Delta^{5,7}$-sterol. $\Delta^{5,8,22}$-, $\Delta^{7,22}$-, zymo made by leaky mutant.
YPD + Nystatin	11 mutants had no $\Delta^{5,7}$-sterol, only $\Delta^{5,8}$ or Δ^8: *erg2* or *erg6*. Rest are leaky *erg2, erg3, erg6*.	*erg2, erg3, erg4, erg6* equally found.	Found *erg2, erg3, erg4, erg5, erg6*. *erg6* is predominant mutant species.
YPD + Filipin	13 to 15 are *erg4* or *erg6*, rest are *erg2* or *erg3*, most make predominantly Δ^8-sterols.	Most mutants examined are *erg5* (37 of 50), rest are *erg2, erg3, erg4* and *erg6*.	Wide range of mutants as above.

[a]Mutagenesis performed following the procedure of Lewis *et al.* (42).
[b]Strains used: W303-1A (Mata *leu2 his3 ura3 trp1 can1-100 ade2*), JC530-4b (Mata *leu2 his4 ura3*), x2180-1A (Mata *SUC2 cup1 gal2*).
[c]YPG: 0.5% yeast extract, 1.0% peptone and 2.0% glycerol; YPD: 0.5% yeast extract, 1.0% peptone and 2.0% dextrose.

sterols in combination with other alterations in sterol biosynthesis, indicating substantial leakiness in the mutants. There were marked differences in the ratios of different sterols in the different mutants. We realize that these experiments are only presumptive of the roles for sterol features on respiration, but they do not provide an interesting opportunity to speculate on the importance of the principal ring features as being important in respiratory development.

CONCLUSIONS

This review emphasizes the varied functions in which sterols participate in the physiology of the yeast cell. As the tools of biophysics, biochemistry, genetics, and molecular biology are increasingly more effectively applied to this field, a convergence of understanding is emerging. Researchers, looking at different aspects of cell physiology, are finding sterols involved in structural, regulatory, and developmental roles. With the abundance of data that is accumulating, a greater appreciation is required for the differences in strains, culture conditions and techniques that are being used in the various laboratories. Explanations for seemingly disparate conclusions can provide an even greater depth of insight into the roles of sterols in the general economy of the cell.

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. R.J. Trumbly for providing the information on *lis1* in advance of its publication. Special thanks are given to Dr. W.D. Nes and Dr. E. Parish for organizing the symposium on sterols at which this paper was originally presented. The original research cited in this paper was conducted with the support of the National Institutes of Health (DK37222) and the North Carolina Agricultural Research Service.

REFERENCES

1. Rodriguez, R.J., Low, C., Bottema, C.D.K., and Parks, L.W. (1985) *Biochim. Biophys. Acta 837*, 336–343.
2. Parks, L.W., Anding, C., and Ourisson, G. (1974) *Eur. J. Biochem. 43*, 451–458.
3. Gaber, R.F., Copple, D.M., Kennedy, B.K., Vida, M., and Bard, M. (1989) *Mol. Cell. Biol. 9*, 3447–3456.
4. McCammon, M.T., Hartmann, M.-A., Bottema, C.D.K., and Parks, L.W. (1984) *J. Bacteriol. 157*, 475–483.
5. Grenson, M., and Crabeel, M. (1970) *J. Bacteriol. 103*, 770–777.
6. Kotyk, A., and Dvorakova, M. (1990) *Folia Microbiol. 35*, 209–217.
7. Welihinda, A.A., Beavis, A.D., and Trumbly, R.J. (1994) *Biochim. Biophys. Acta 1193*, 107–117.
8. Bard, M., Lees, N.D., Burrows, L.S., and Kleinhans, F.W. (1978) *J. Bacteriol. 135*, 1146–1148.
9. Vogel, J.P., Lee, J.N., Kirsch, D.R., Rose, M.D., and Sztul, E.S. (1993) *J. Biol. Chem. 268*, 3040–3042.
10. Graham, T.R., Scott, P.A., and Emr, S.D. (1993) *Embo. J. 12*, 869–877.
11. Shah, N., and Klausner, R.D. (1993) *J. Biol. Chem. 268*, 5345–5348.
12. Tomeo, M.E., Fenner, G., Tove, S.R., and Parks, L.W. (1992) *Yeast 8*, 1015–1024.
13. Pryer, N.K., Wuestehube, L.J., and Schekman, R. (1992) *Ann. Rev. Biochem. 61*, 471–516.
14. Pelham, H.R.B. (1989) *Ann. Rev. Cell. Biol. 5*, 1–23.
15. Semenza, J.C., Hardwick, K.G., Dean, N., and Pelham, H.R.B. (1990) *Cell 61*, 1349–1357.
16. Hardwick, K.G., and Pelham, H.R.B. (1994) *Yeast 10*, 265–269.
17. Clausen, J.K., Christiansen, K., Jensen, P.K., and Behnke, O. (1974) *FEBS Lett. 43*, 176–179.
18. Atkinson, D.E. (1977) *Cellular Energy Metabolism and Its Regulation*, Academic Press, New York.
19. Parks, L.W. (1978) *Crit. Rev. Microbiol. 6*, 301–341.
20. Akhtar, M., Brooks, W.A., and Watkinson, I.A. (1969) *Biochem. J. 115*, 135–137.
21. Alexander, K., Akhtar, M., Boar, R.B., McGhie, J.F., and Barton, D.H.R. (1972) *J. Chem. Soc. Chem. Commun.*, 383–385.
22. Bottema, C.K., and Parks, L.W. (1978) *Biochim. Biophys. Acta 531*, 301–307.
23. Hays, P.R., Neal, W.D., and Parks, L.W. (1977) *Antimicrob. Agents Chemother. 12*, 185–191.
24. Hays, P.R., Parks, L.W., Pierce, H.D., and Oehlschlager, A.C. (1977) *Lipids 12*, 666–668.
25. Bailey, R.B., Hays, P.R., and Parks, L.W. (1976) *J. Bacteriol. 128*, 730–734.
26. Baloch, R.I., Mercer, E.I., Wiggins, T.E., and Baldwin, B.C. (1984) *Phytochem. 23*, 2219–2226.
27. Marcireau, C., Guilloton, M., and Karst, F. (1990) *Antimicrob. Agents Chemother. 34*, 989–993.
28. Lorenz, R.T., and Parks, L.W. (1991) *Antimicrob. Agents Chemother. 35*, 1532–1537.
29. Lorenz, R.T., and Parks, L.W. (1992) *DNA Cell Biol. 11*, 685–692.
30. Steel, C.C., Baloch, R.I., Mercer, E.I., and Baldwin, B.C. (1989) *Pestic. Biochem. Physiol. 33*, 101–111.
31. Crowley, J.H., Lorenz, R.T., and Parks, L.W. (1994) *Antiomicrob. Agents Chemother. 38*, 1004–1007.
32. Grenson, M. (1969) *Eur. J. Biochem. 11*, 249–260.
33. Jund, R., and Lacroute, F. (1970) *J. Bacteriol. 102*, 607–615.
34. Osumi, T., Nishino, T., and Katsuki, H. (1979) *J. Biochem. (Tokyo) 85*, 819–826.
35. Rodriguez, R.J., Taylor, F.R., and Parks, L.W. (1982) *Biochem. Biophys. Res. Commun. 106*, 435–441.
36. Rodriguez, R.J., and Parks, L.W. (1983) *Arch. Biochem. Biophys. 225*, 861–871.
37. Lorenz, R.T., Casey, W.M., and Parks, L.W. (1989) *J. Bacteriol. 171*, 6169–6173.
38. Smith, S.J., and Parks, L.W. (1993) *Yeast 9*, 1177–1187.
39. Arthington, B.A., Bennett, L.G., Skatrud, P.L., Guynn, C.J., Burbuch, R.J., Ulbright, C.E., and Bard, M. (1991) *Gene 102*, 39–44.
40. Lewis, T.A., Taylor, F.R., and Parks, L.W. (1985) *J. Bacteriol. 163*, 199–207.
41. Shinabarger, D.L., Keesler, G.A., and Parks, L.W. (1989) *Steroids 53*, 607–623.
42. Lewis, T.A., Taylor, F.R., and Parks, L.W. (1985) *J. Bacteriol. 163*, 199–207.

[Received June 20, 1994, and in revised form January 13, 1995; Revision accepted January 13, 1995]

Identification of the Active Site of Vertebrate Oxidosqualene Cyclase[1]

Ikuro Abe and Glenn D. Prestwich*

Department of Chemistry, University at Stony Brook, Stony Brook, New York 11794-3400

ABSTRACT: Active site mapping of rat liver oxidosqualene cyclase (OSC), a 78 kDa membrane-bound enzyme, was carried out using the mechanism-based irreversible inhibitor, [^{3}H]29-methylidene-2,3-oxidosqualene. The amino acid sequence of the radiolabeled CNBr peptide fragment showed unexpectedly high similarity to the yeast OSC, plant OSC, and bacterial squalene cyclases. Further, radio analysis established that the two adjacent Asp residues in the highly conserved region (*Asp-Asp*-Thr-Ala-Glu-Ala, or DDTAEA) were equally labeled by the irreversible inhibitor. This result provided the first information on the structural details of the active site of OSC, and showed for the first time the ancient lineage of this vertebrate enzyme to ancestral eukaryotic and prokaryotic cyclases. Interestingly, the covalently-modified DDXX(D/E) sequence of rat liver OSC showed surprising similarity to the putative allylic diphosphate binding site sequence of other terpene cyclases and prenyl transferases. The Asp-rich motif may act as a point charge to stabilize incipient cationic charge.

Lipids 30, 231–234 (1995).

The enzymatic cyclization of oxidosqualenes is the most remarkable step in the biosynthesis of sterols and triterpenes (1). In a single step, an acyclic polyene is converted by a specific enzyme to either a tetracyclic or pentacyclic triterpene. In the case of lanosterol formation, (3*S*)-oxidosqualene first adopts a prechair–boat–chair conformation. Then protonation of the epoxide initiates a cascade of ring-forming reactions and backbone rearrangements that proceed through rigidly held carbocationic intermediates (2–6). Several oxidosqualene cyclases (OSC) (EC 5.4.99.7) have been purified to homogeneity from vertebrate (7–9), plant (10–13), and yeast sources (14). Recently, three OSC enzymes have been cloned and sequenced from two fungal sources, *Saccharomyces cerevisiae* (15,16) and *Candida albicans* (17–19) and, from one plant, *Arabidoposis thaliana* (20). The predicted molecular masses ranged from 80 to 90 kDa, and the cDNA sequences predicted proteins with significant sequence similarity. In addition, sequence comparisons of these cyclases with bacterial squalene cyclases (SC) revealed an existence of a highly-conserved repetitive motif rich in aromatic amino acids (the QW motif) (21,22).

Recent work from our laboratories on active site mapping of rat liver OSC using 29-methylidene-2,3-oxidosqualene (29-MOS), the first mechanism-based irreversible inactivator of vertebrate OSC, will be summarized in this paper. 29-MOS was first prepared in racemic form and showed potent inhibition of OSC. An IC_{50} value of 0.5 μM, an apparent K_i value of 4.4 μM, a k_{inact} value of 221 min^{-1}, and a partition ratio of 3.8 (inactivations per turnover) were obtained for pig liver OSC (23). Incubation of 0.1 μM 29-MOS with pig liver OSC gave a polycyclic product in 30% yield; however, at [29-MOS] > K_i, complete inactivation precluded isolation of this product (23). A new synthesis of the enantiomerically-enriched substrate, [^{3}H](3*S*)-29-MOS, by the Sharpless asymmetric dihydroxylation reaction (24–26) was achieved recently (27). Several other 26- and 29-functionalized 2,3-oxidosqualene derivatives were found to be slightly less active than 29-MOS (28).

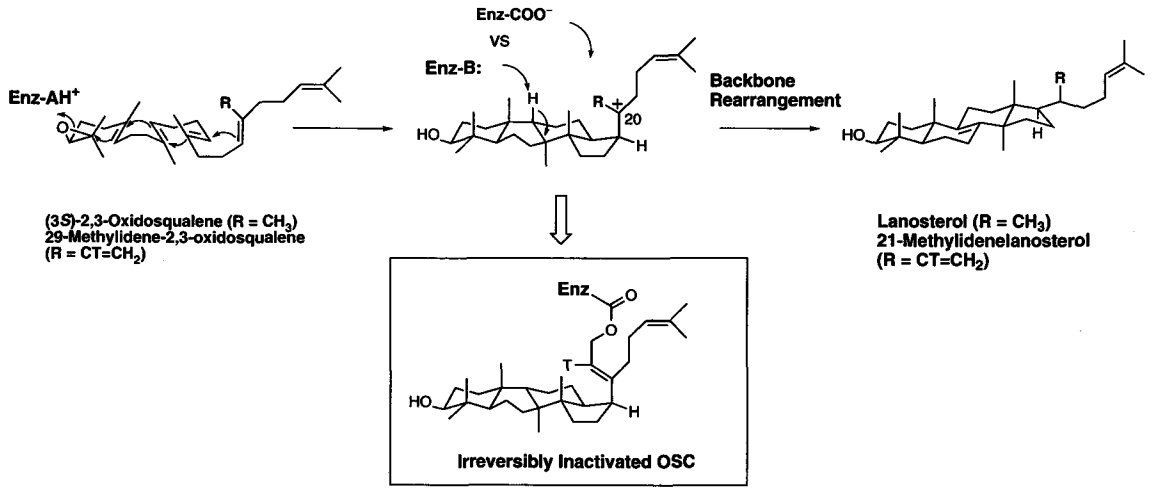
Vertebrate OSC enzymes can be efficiently and specifically labeled with tritium-labeled 29-MOS (8). For a typical affinity labeling experiment, enzyme preparations were first incubated with [^{3}H]29-MOS (1.0 μM, 2.3 Ci/mmol) for 15 min at 37°C. Next, the proteins were separated by denaturing polyacrylamide gel electrophoresis (SDS–PAGE), and the covalently-modified protein bands in fluorophore-enhanced, dried gels were visualized on X-ray film by fluorescence autoradiography. For each enzyme source, a single radiolabeled protein band was observed having a molecular mass between 70 and 80 kDa (rat, 78 kDa; pig, 75 kDa; dog, 73 kDa; and human, 73 kDa). The proposed mechanism of inhibition by 29-MOS involves initial cyclization to the 21-methylidene-protosterol cation as proposed for lanosterol formation. However, we have postulated that instead of backbone rearrangement, this allylic cation can be trapped by an active-site nucleophile, resulting in irreversible inactivation of the enzyme (Scheme 1). Moreover, this hypothesis also predicts that the structures of the released and enzyme-bound tetracyclic products are different; 21-methylidene lanosterol should be released, while the covalent moiety attached to the enzyme

[1]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed.

Abbreviations: 29-MOS, 29-methylidene-2,3-oxidosqualene; OSC, oxidosqualene cyclase; SC, squalene cyclase; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

Copyright © 1995 by AOCS Press



SCHEME 1

should retain the unrearranged protosterol skeleton. Verification of these structures is in progress.

In contrast, yeast and plant OSC enzymes were not labeled even under forcing conditions, and [^{3}H]29-MOS was recovered unchanged by chromatography (8). Because 29-hydroxy-2,3-oxidosqualene is quantitatively converted to 21-hydroxylanosterol by yeast OSC (29), it seemed most likely that 29-MOS would be accepted as a substrate. The initially formed 21-methylidene protosterol cation could be stabilized by the enzyme, preventing backbone rearrangement to the cyclization products. In this case, instead of covalent bond formation, the stabilization would probably be attained by an electrostatic interaction. As a result, 29-MOS appears to act as a slow tight-binding inhibitor; and an IC_{50} value of 1.5 μM, an apparent K_i value of 1.5 μM, and a k_{inact} value of 0.088 min^{-1} were obtained for yeast OSC (8).

In order to localize the 29-MOS binding site region of rat liver OSC, purified enzyme was labeled with [^{3}H]29-MOS and then selectively cleaved adjacent to Met residues with CNBr (30). A single radioactive 6 kDa peptide fragment was separated by Tricine SDS–PAGE. After electrophoretic transfer to a polyvinylidene difluoride membrane, the labeled peptide was sequenced by Edman degradation (30). The sequence of the first 30 amino acids of the peptide showed unexpectedly high similarity to the other eukaryotic sterol and terpene cyclases: 50% identity with Arg^{439}–Val^{468} of yeast OSC (15,16), 50% identity with Arg^{433}–Val^{462} of *C. albicans* OSC (18,19), 63% identity with Ser^{466}–Leu^{495} of plant *A. thaliana* OSC (cycloartenol synthase) (20), and to the bacterial SC enzymes, 33% identity with Lys^{356}–Leu^{384} of *Alicyclobacillus* (formerly *Bacillus*) *acidocaldarius* SC (31), 27% identity with Arg^{375}–Ala^{404} of *Zymomonas mobilis* SC (32) (Fig. 1). This result provides the first information on the structural details of the active site of OSC and demonstrates the ancient lineage of this vertebrate enzyme with respect to the ancestral eukaryotic and prokaryotic cyclases.

Furthermore, radioanalysis clearly established that the two adjacent Asp residues in the highly-conserved region (*Asp*-Thr-Ala-Glu-Ala, or DDTAEA) were equally labeled by the irreversible inhibitor (30). The initially formed 21-methylidene-protosterol cation would be trapped by nucleophilic attack by the carboxyl group of one of the two adjacent Asp residues, resulting in ester bond formation. The two Asp residues appear to be equally accessible to the methylidene-extended protosterol cation. In the case of yeast or plant OSC enzymes, the appropriate nucleophilic residues seem to either be absent or not to have access to the allylic cation. Indeed, each of these enzymes has a Cys residue in the second position, giving a characteristic DCTAEA motif. Recently, rat liver OSC was cloned and sequenced in our laboratory (Abe, I., and Prestwich, G.D., unpublished data). According to the deduced amino acid sequence of this cDNA, the 29-MOS binding site has the DCTAEA sequence instead of DDTAEA. Isozymes of this enzyme with the DDTAEA sequence may exist, or there may be sequencing anomalies associated with a lipid-modified amino acid. We are exploring these two possibilities.

Interestingly, the covalently-modified DDTAEA sequence of rat liver OSC showed surprising similarity to the known consensus sequence, Asp-Asp-Xaa-Xaa-Asp (the "DDXXD" motif) found in diterpene cyclases (33), sesquiterpene cyclases (34–37), and monoterpene cyclases (33), as well as in several prenyl transferases (38–40) (Fig. 1). Each of these enzymes uses an allylic diphosphate as a substrate and divalent metal ions as a co-factor. Thus, this aspartate-rich domain has been implicated in catalyzing the ionization of the allylic diphosphate to the reactive allylic cation, presumably by coordination *via* an Mg^{2+}-diphosphate bridge. Recent site-directed mutagenesis experiments of the conserved aspartate residues of farnesyl–diphosphate synthase suggest that the first Asp residue in this domain is important in catalysis (41–43). The high degree of sequence conservation of the 29-MOS binding site in triterpene and sterol cyclases supports a catalytic or structural function for this domain. Stabilization of tertiary or allylic carbocations may be the true raison d'être for the DDXX(D/E) motif. We present this hypothesis in

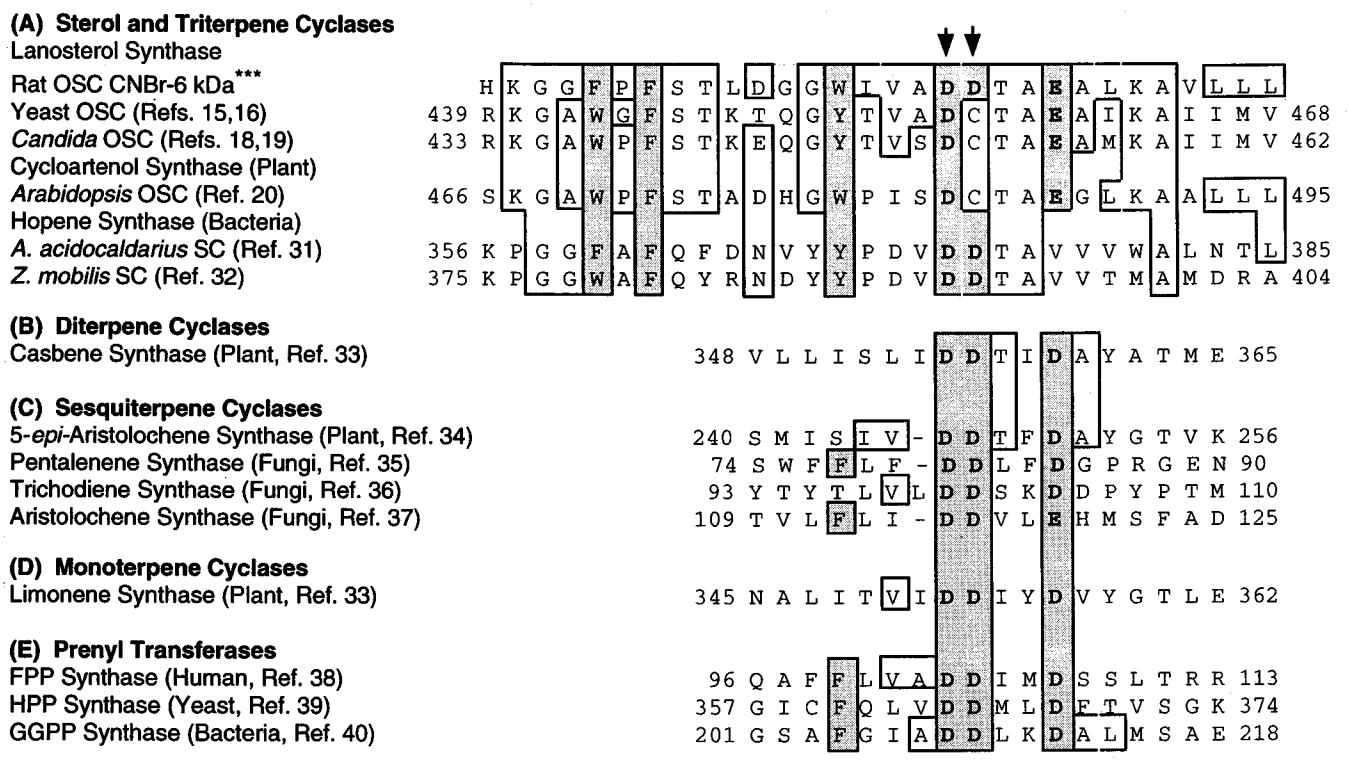
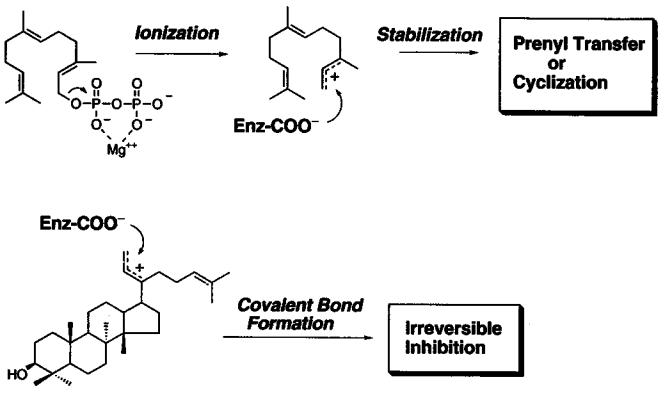


FIG. 1. Comparison of the DDXX(D/E) motif in the predicted amino acid sequences of terpene biosynthetic enzymes. OSC, oxidosqualene cyclase; SC, squalene cyclase.



SCHEME 2

Scheme 2, and we suggest that rather than assisting the departure of the pyrophosphate, the Asp-rich motif may act as a point charge to stabilize incipient cationic charge. For transferases and other cyclases, this corresponds to stabilization of the transition state to a high-energy intermediate cation at the initiation of the reaction, or perhaps during cyclization. For OSC, the Asp-rich motif would stabilize a "stopping point" in the cyclization pathway, i.e., after tetracycle formation but prior to backbone rearrangement. Very recently, Poralla *et al.* (44) reported that a point mutation of the first Asp residue of the DDTAVV motif of bacterial SC (*A. acidocaldarius*) can cause almost complete loss of the cyclase activity. Additional single mutations in cyclases will provide further tests for this hypothesis. Finally, it should be noted that the existence of such a consensus sequence in the terpene and sterol biogenesis enzymes is very interesting, considering the molecular evolution of these enzymes.

ACKNOWLEDGMENTS

The authors are indebted to T. Fischer at the Center of Analysis and Synthesis of Macromolecules (Stony Brook) for Edman peptide sequencing and Dr. X.-y. Xiao for synthesis of the [^{3}H]29-MOS used in this study. This work was supported by the Center for Biotechnology/New York State Science and Technology Foundation, Sandoz Research Institute and the National Institutes of Health (GM 44836).

REFERENCES

1. Abe, I., Rohmer, M., and Prestwich, G.D. (1993) *Chem. Rev. 93*, 2189–2206.
2. Eschenmoser, A., Ruzicka, L., Jeger, O., and Arigoni, D. (1955) *Helv. Chim. Acta 38*, 1890–1904.
3. Stork, G., and Burgstahler, A.W. (1955) *J. Am. Chem. Soc. 77*, 5068–5077.
4. van Tamelen, E.E. (1982) *J. Am. Chem. Soc. 104*, 6480–6481.
5. Corey, E.J., Virgil, S.S., and Sarshar, S. (1991) *J. Am. Chem. Soc. 113*, 8171–8172.
6. Corey, E.J., and Virgil, S.C. (1991) *J. Am. Chem. Soc. 113*, 4025–4026.
7. Kusano, M., Abe, I., Sankawa, U., and Ebizuka, Y. (1991) *Chem. Pharm. Bull. 39*, 239–241.

8. Abe, I., Bai, M., Xiao, X.-y., and Prestwich, G.D. (1992) *Biochem. Biophys. Res. Commun. 187*, 32–38.
9. Moore, W.R., and Schatzman, G.L. (1992) *J. Biol. Chem. 267*, 22003–22006.
10. Abe, I., Ebizuka, Y., and Sankawa, U. (1988) *Chem. Pharm. Bull. 36*, 5031–5034.
11. Abe, I., Sankawa, U., and Ebizuka, Y. (1989) *Chem. Phar. Bull. 37*, 536–538.
12. Abe, I., Ebizuka, Y., Seo, S., and Sankawa, U. (1989) *FEBS Lett. 249*, 100–104.
13. Abe, I., Sankawa, U., and Ebizuka, Y. (1992) *Chem. Pharm Bull. 40*, 1755–1760.
14. Corey, E.J., and Matsuda, S.P. (1991) *J. Am. Chem. Soc. 113*, 8172–8174.
15. Corey, E.J., Matsuda, S.P.T., and Bartel, B. (1994) *Proc. Natl. Acad. Sci. USA 91*, 2211–2215.
16. Shi, Z., Buntel, C., and Griffin, J.H. (1994) *Proc. Natl. Acad. Sci. USA 91*, 7370–7374.
17. Kelly, R., Miller, S.M., Lai, M.H., and Kirsch, D.R. (1990) *Gene 87*, 177–183.
18. Buntel, C.J., and Griffen, J.H. (1992) *J. Am. Chem. Soc. 114*, 9711–9713.
19. Roessner, C.A., Min, C., Hardin, S.H., Harris-Haller, L.W., McCollum, J.C., and Scott, A.I. (1993) *Gene 127*, 149–150.
20. Corey, E.J., Matsuda, S.P.T., and Bartel, B. (1993) *Proc. Natl. Acad. Sci. USA 90*, 11628–11632.
21. Poralla, K., Hewelt, A., Prestwich, G.D., Abe, I., Reipen, I., and Sprenger, G. (1994) *Trends Biochem. Sci. 19*, 157–158.
22. Poralla, K. (1994) *Bioorg. Med. Chem. Lett. 4*, 285–290.
23. Xiao, X.-y., and Prestwich, G.D. (1991) *J. Am. Chem. Soc. 13*, 9673–9674.
24. Sharpless, K.B., Amberg, W., Bennani, Y.L., Crispino, G.A., Hartung, J., Jeong, K.-S., Kwong, H.-L., Morikawa, K., Wang, Z.-M., Xu, D., and Zhang, X.-L. (1992) *J. Org. Chem. 57*, 2768–2771.
25. Crispino, G.A., and Sharpless, K.B. (1992) *Tetrahedron Lett. 33*, 4273–4274.
26. Crispino, G.A., Ho, P.T., and Sharpless, K.B. (1993) *Science 259*, 64–66.
27. Madden, B.A., and Prestwich, G.D. (1994) *J. Org. Chem. 59*, 5488–5489.
28. Abe, I., Tomesch, J.C., Wattanasin, S., and Prestwich, G.D. (1994) *Nat. Prod. Rep. 11*, 279–302.
29. Xiao, X.-y., and Prestwich, G.D. (1991) *Tetrahedron Lett. 32*, 6843–6846.
30. Abe, I., and Prestwich, G.D. (1994) *J. Biol. Chem. 269*, 802–804.
31. Ochs, D., Kaletta, C., Entian, K.-D., Beck-Sickinger, A., and Poralla, K. (1992) *J. Bacteriol. 174*, 298–302.
32. Reipen, I., Sprenger, G., and Poralla, K. (1993) *GenBank Data Base*, accession no. X73561.
33. Colby, S.M., Alonso, W.R., Katahira, E.J., McGarvey, D.J., and Croteau, R. (1993) *J. Biol. Chem. 268*, 23016–23024.
34. Facchini, P.J., and Chappel, J. (1992) *Proc. Natl. Acad. Sci. USA 89*, 11088–11092.
35. Cane, D.E., Sohng, J.-K., Lamberson, C.R., Rudnicki, S.M., Wu, Z., Lloyd, M.D., Oliver, J.S., and Hubbard, B.R. (1994) *Biochemistry 33*, 5846–5857.
36. Hohn, T.M., and Beremand, P.D. (1989) *Gene 79*, 131–138.
37. Proctor, R.H., and Hohn, T.M. (1993) *J. Biol Chem. 268*, 4543–4548.
38. Sheares, B.T., White, S.S., Molowa, D.T., Chan, K., Ding, V.D.H., Kroon, P.A., Bostedor, R.G., and Karkas, J.D. (1989) *Biochemistry 28*, 8129–8135.
39. Ashby, M.N., and Edwards, P.A. (1990) *J. Biol. Chem. 265*, 13157–13164.
40. Math, S.K., Hearst, J.E., and Poulter, C.D. (1992) *Proc. Natl. Acad. Sci. USA 89*, 6761–6764.
41. Marrero, P.F., Poulter, C.D., and Edwards, P.A. (1992) *J. Biol. Chem. 267*, 21873–21878.
42. Joly, A., and Edwards, P.A. (1993) *J. Biol. Chem. 268*, 26983–26989.
43. Song, L., and Poulter, C.D. (1994) *Proc. Natl. Acad. Sci. USA 91*, 3044–3048.
44. Poralla, K., Ochs, D., and Feil, C. (1994) *INFORM 5*, 470.

[Received July 14, 1994, and in revised form December 23, 1994; Revision accepted December 27, 1994]

2,3-Oxidosqualene Cyclase: From Azasqualenes to New Site-Directed Inhibitors[1]

Luigi Cattel*, Maurizio Ceruti, Gianni Balliano, Franca Viola, Giorgio Grosa, Flavio Rocco and Paola Brusa

Istituto di Chimica Farmaceutica Applicata, Facoltà di Farmacia, 10125 Torino, Italy

ABSTRACT: 2,3-Oxidosqualene cyclases (OSC) are enzymes which convert 2,3-oxidosqualene (OS) into polycyclic triterpenoids such as lanosterol, cycloartenol, and α- and β-amyrin. Our interest in the study of OSC is the development of new OSC inhibitors for potential use as hypocholesterolemic, antifungal, or phytotoxic drugs. In particular, we describe the biological activity and the mechanism of a series of acyclic azasqualene derivatives mimicking the C-2, C-8, and C-20 carbonium ions formed during OS cyclization. Some of these carbonium ion analogues are very promising as specific hypocholesterolemic agents. The toxicity, the biodistribution, and the pharmacokinetics of different azasqualene derivatives in mice are also presented. In order to obtain new, site-directed irreversible inhibitors of OSC, a series of squalene derivatives containing functional groups that can link covalently to an active-site thiol group was designed. Among these compounds, squalene maleimide was the most active toward mammalian OSC, whereas squalene Ellman behaved as an irreversible inhibitor of OSC from yeast.

Lipids 30, 235–246 (1995).

2,3-Oxidosqualene cyclases (OSC) are key enzymes in the biosynthesis of animal, plant, and fungal sterols. They catalyze the conversion of (*3S*)-2,3-oxidosqualene **1** to lanosterol **8** (Scheme 1) in mammals and fungi and to cycloartenol in algae and higher plants (1–7). Moreover, OSC are responsible for the formation of a variety of tetra- or pentacyclic triterpenoids found in higher plants (8,9). In some bacteria and protozoa, squalene can be directly cyclized to tetrahymanol-type pentacyclic triterpenoids (7).

The enzymatic cyclization of 2,3-oxidosqualene (OS) **1** starts with an initial protonation of the oxirane ring, which is accomplished by a suitable electrophilic residue present at the active site of the enzyme to give a C-2 carbonium ion intermediate **2**, followed by cyclization to a series of discrete, conformationally rigid carbonium ion intermediates such as the C-10 monocyclic intermediate **3**, the C-8 bicyclic **4**, the C-13 tricyclic **5**, and the C-20 tetracyclic carbonium ion **6** or **7** (10–19) (Scheme 1). These intermediates, which are also called high-energy intermediates (HEI) (10,20), undergo further rearrangement to form lanosterol **8** in animals and yeasts or cycloartenol in higher plants.

Our interest in the study of OSC is the development of new inhibitors for potential use as hypocholesterolemic, antifungal, or phytoxic agents (15,21). Inhibition of OSC has constituted an attractive approach for the design and synthesis of drugs, as this step is beyond the synthesis of other triterpenoid intermediates, such as dolichol, ubiquinone, and farnesylated proteins, which have important physiological functions including those related to cell division (22).

A general strategy for the design of new inhibitors of OSC has been to mimic the previously described carbonium ion intermediates by replacing the positively charged carbonium ion with a nitrogen (protonated at physiological pH) (23–27). These inhibitors are being thought of as analogues of the enzyme-bound HEI (10).

Several laboratories have synthesized cyclic aza derivatives (Scheme 2) with a nitrogen corresponding to the C-10 HEI, such as in **9**, **10**, and analogues, or to the C-8 HEI, such as the azadecalin derivatives **11** and **12** (27–32). These mono- and bicyclic derivatives were shown to be potent inhibitors of OSC, whereas the tricyclic ones, such as **13**, showed only modest inhibition of OSC *in vitro* (31). It is worth pointing out that some uncharged species, such as the piperidine amide **14**, the decalin amide **15**, or the thioamide derivative **16**, are also potent inhibitors of OSC (33,34). Finally, a series of sulfur-containing oxidosqualene analogues **17** and **18** (Scheme 3) showed significant inhibition of the mammalian OSC (28).

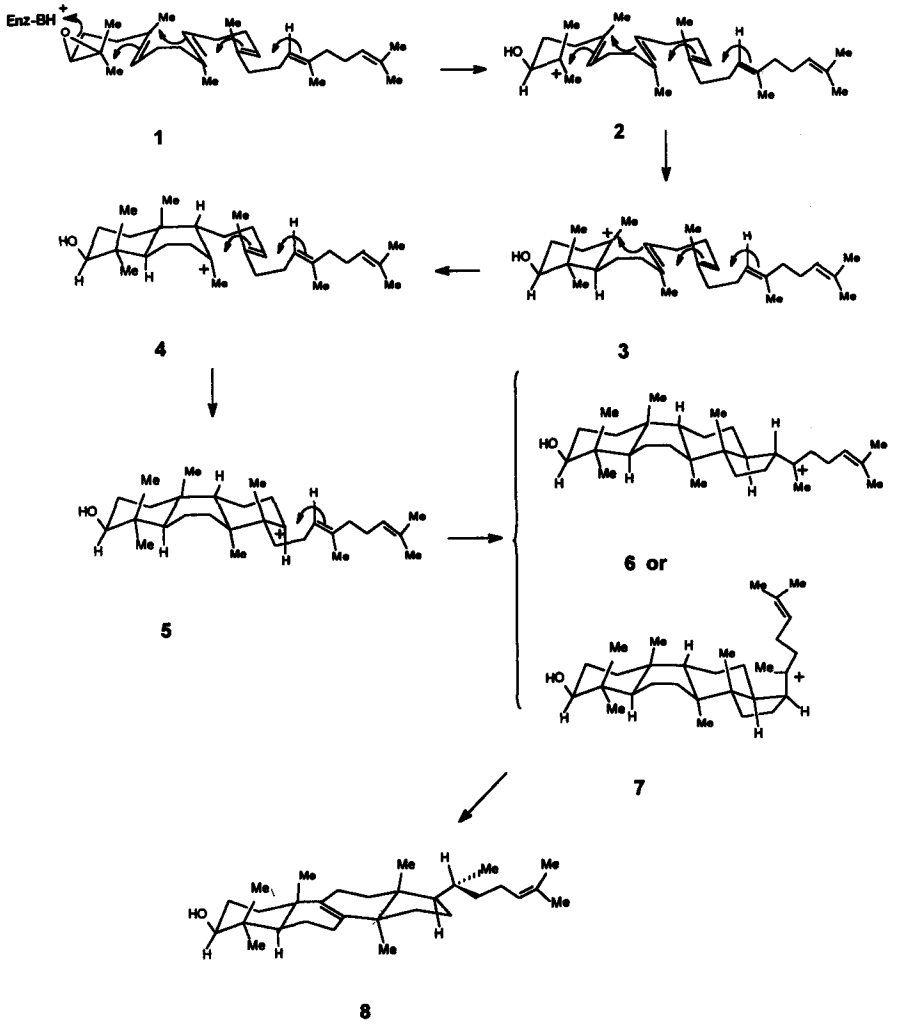
Our strategy has been to design acyclic aza squalenoid derivatives **21–23** (Scheme 4) possessing a nitrogen atom corresponding to the carbonium ions or HEIs **2, 4,** and **7** arising from cyclization of OS **1**. We believe that these acyclic compounds are conformationally more flexible than the cyclic ones, and thus they should be less likely to interfere with

[1]Based on a paper presented at the symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at Istituto di Chimica Farmaceutica Applicata, Facoltà di Farmacia, Corso Raffaello 31, 10125 Torino, Italy.

Abbreviations: CNDT, 3-carboxy-4-nitrophenyldithio-1,1′,2-*tris*-norsqualene; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic) acid; HEI, high energy intermediate; HPLC, high-performance liquid chromatography; IC_{50}, the concentration of inhibitor which reduces the enzymatic conversion by 50%; ip, intraperitoneal; iv, intravenous; LD_{50}, dose that kills 50% of animals; OSC, 2,3-oxidosqualene cyclase; OS, 2,3-oxidosqualene; TLC, thin-layer chromatography; TNB, thionitrobenzoate.

Copyright © 1995 by AOCS Press



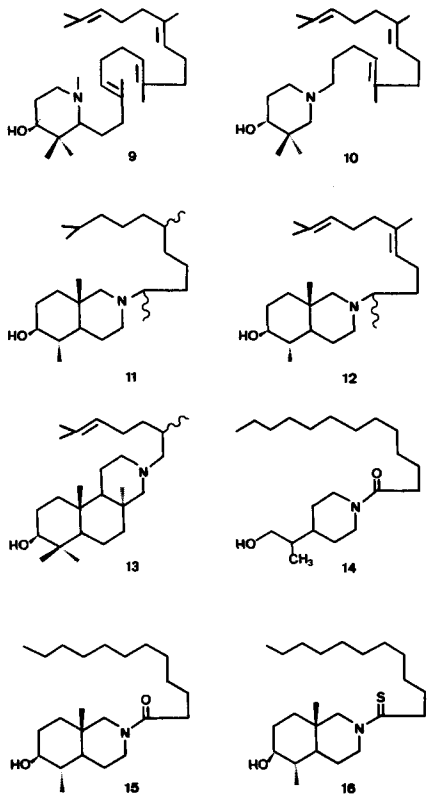
SCHEME 1

other sterol biosynthetic enzymes, such as the sterol $\Delta^8 \rightarrow \Delta^7$ isomerase or sterol Δ^{14} reductase, which are sometimes inhibited by the cyclic inhibitors (32–37). Some of these inhibitors can be considered as product state analogues, like the known OSC inhibitor 4,4,10β-trimethyl-*trans*-decal-3β-ol **19** (Scheme 3) (35).

Detailed kinetic studies with microsomal or partially solubilized OSC indicated that both the acyclic and the cyclic aza derivatives apparently behaved as noncompetitive inhibitors (38–40). It has been postulated (31) that cyclic inhibitors such as the aza tricyclic compounds mimicking HEI with structures very different from those of substrates or products could be slow-binding inhibitors. So far only some noncharged inhibitors, such as the 19-oxa vinylether analogue of OS or the bicyclic amide **15**, were shown to be competitive inhibitors of OSC (41). Usually, such kinetic studies are difficult to carry out with the OSC membrane-bound enzymes associated with the microsomal fraction, or solubilized and partially purified OSC, both of which can be considered highly heterogeneous.

The first mechanism-based irreversible inhibition of OSC was recently reported by Xiao and Prestwich (42). 29-Methylidene-2,3-oxidosqualene **20** (Scheme 3) was a very potent inactivator of pig liver OSC, showing an IC_{50} (the concentration of inhibitor which reduces the enzymatic conversion by 50%) value of 0.5 μM, an apparent K_i of 4.4 μM, a $k_{inactiv}$ of 221 min^{-1}, and a partition ratio of 3.8. By affinity labeling experiments, it was shown that **20** specifically labeled vertebrate OSC, whereas neither yeast or plant OSC was labeled (43). Covalently-modified peptide fragments were successively isolated from purified rat liver OSC and sequenced. The labeled peptide contained a DDTAE motif characterized by the presence of two adjacent aspartate residues (44).

The synthesis of new, site-directed irreversible inhibitors is, in our view, a very interesting task as it provides new tools for preparing potent therapeutic agents, as well as for modifying the active site, to gain mechanistic insights into the mode of action of the enzyme. We are particularly interested in the design of specific thiol modifying agents with a squalene-type structure, as several authors have demonstrated an irreversible inhibition of OSC from different sources by some thiol reagents such as *p*-chloromercuribenzenesulfonic acid

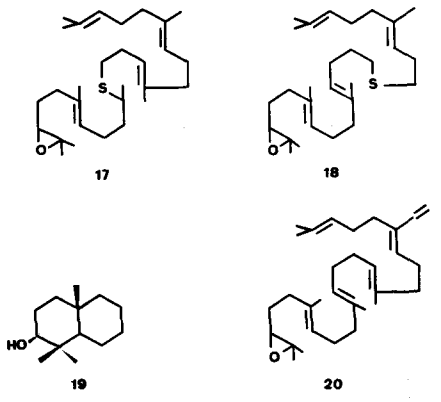


SCHEME 2

and *N*-ethylmaleimide in hog liver, yeast, and pea seedlings cyclase (45–49). Similar results were reported by Poralla for squalene–hopene cyclase from *Bacillus acidocaldarius* (50).

MATERIALS AND METHODS

IC_{50} values on OS–lanosterol cyclase activity were determined in microsomal preparations from rat liver, pig liver, *Saccharomyces cerevisiae* and *Candida albicans* and on partially purified OS cyclase of pig liver. IC_{50} values on squalene epoxidase activity were determined in microsomal preparations from rat and pig liver.

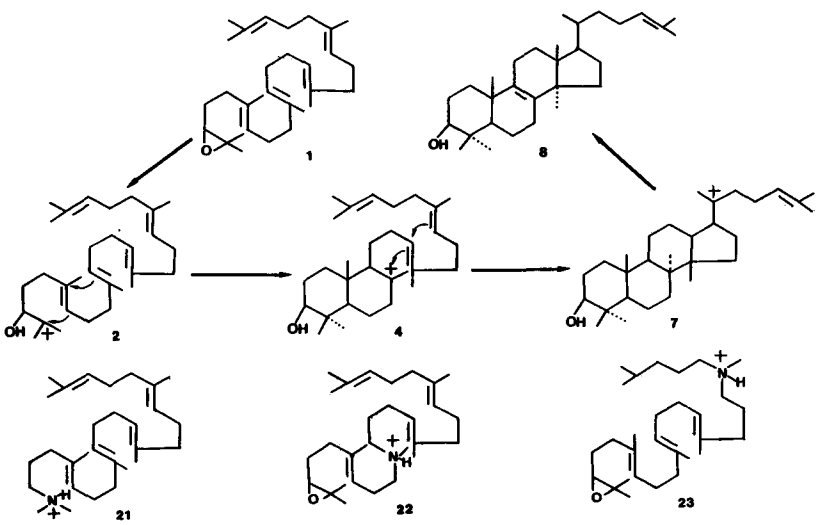


SCHEME 3

Microsomes of rat and pig liver and of *S. cerevisiae* were prepared according to the methods previously described (38,41,51,52). *Candida albicans* microsomes were kindly provided by LEPETIT (Gerenzano, VR, Italy).

Pig liver OSC was purified according to the method previously described (38).

Assay of mammalian oxidosqualene cyclase activity. The substrate and the insoluble inhibitors were added to the test tubes as organic solutions in $CHCl_3$ in the presence of 0.05% (wt/vol) Tween 80, and the solvent was evaporated under nitrogen before addition of the aqueous components of the mixture. OSC activity was determined by incubating the microsomal suspension (5 mg protein for rat liver or 1 mg for pig liver) or the solubilized enzyme preparation (250 µL, 0.1 mg protein) for 30 min at 45°C with [3-^{3}H](*R,S*)-OS (100,000 dpm) diluted with (*R,S*)-OS (50 nmol), 0.1 M K/K phosphate



SCHEME 4

buffer, pH 8, containing 1 mM EDTA, Tween 80 (final concentration, 0.05% wt/vol), the inhibitor (when present) and 0.05% Emulphogene for solubilized enzyme (final volume, 1 mL). The reaction was stopped, and the amount of lanosterol was determined as previously described (41).

IC_{50} values were determined at 50 μM substrate in the presence of different concentrations of the inhibitors (38).

Assay of yeast oxidosqualene cyclase activity. The substrate OS, detergent and inhibitors, when present, were added to the test tubes as organic solutions. After evaporation of the solvent under N_2, the products were emulsified by stirring vigorously with a minimum of buffer. When not otherwise indicated, the reaction mixture contained (in a volume of 0.5 mL): [3-^{3}H](*R,S*)-OS (100,000 dpm) diluted with (*R,S*)-OS (25 nmol), Tween 80, 0.01% (wt/vol), 0.5 mM dithiothreitol, 0.1 M phosphate buffer, pH 7.1, and 1.5 mg microsomal protein. The reaction mixture was incubated for 30 min at 35°C in a shaking water-bath. Boiled microsomes were used as a blank. The reaction was stopped by adding 1 mL of KOH 15% (wt/vol) in methanol. Tubes were capped and heated at 80°C for 30 min. The nonsaponifiable lipids were extracted (51) and fractionated together with authentic samples of OS and lanosterol by thin-layer chromatography (TLC) on silica gel plates developed with CH_2Cl_2. Areas corresponding to OS and lanosterol were visualized under ultraviolet light with berberine. Fractions were scraped off and counted for radioactivity in a Beckman (Fullerton, CA) LS 5000 liquid scintillator (53).

Isotope counting and activity calculations were done as described (51).

Assay of mammalian squalene epoxidase activity. Squalene epoxidase activity was determined in rat and pig liver microsomes in the presence of supernatant fraction S_{100} and of the OSC inhibitor, 3β-(β-dimethylaminoethoxy)androst-5-en-17-one (U-14226A).

The substrate and the insoluble inhibitors were added to the test tubes as organic solutions in $CHCl_3$, in the presence of 0.05% (wt/vol) Tween 80, and the solvent was evaporated under nitrogen before addition of the aqueous components of the mixture. The reaction mixture contained (in the final volume of 1 mL): [^{3}H]squalene (20,000 dpm) diluted with squalene (20 nmol), Tween-80 (final concentration, 0.05% wt/vol), 0.1 M K/K phosphate buffer, pH 7.4, containing 1 mM EDTA, microsomes (5 mg protein), S_{100} (10 mg protein), U-14226A (50 μM), $NADP^+$ (2 mM), glucose-6-P (5 mM), glucose-6-P dehydrogenase (1 unit), and $MgCl_2 \cdot 6H_2O$ (5 mM). Incubations lasted 30 min at 37°C. The reaction was stopped by the addition of 1 mL of 10% ethanolic KOH followed by saponification for 30 min at 80°C. Extraction and chromatographic procedures similar to those of the OSC assay were used. After developing the TLC plate in CH_2Cl_2, the areas corresponding to authentic squalene and OS were scraped off and the fractions counted for radioactivity in a Beckman LS 5000 liquid scintillator. The enzymatic activity was expressed as nanomoles of OS formed per hour (52).

Inhibition of cholesterol biosynthesis in 3T3 fibroblast and in HEP G2 hepatoma cell cultures. Cells derived from semi-confluent cultures were seeded at about 5×10^5 cells in 17-mm wells in 1 mL of Dulbecco's modified Eagle's medium containing 10% of lipid-free fetal calf serum and were grown for 36 h at 37°C in a humidified incubator under 5% CO_2. After 4 h of preincubation in the presence or absence of inhibitors, [^{14}C]acetate (1 μCi, 0.018 μmol) was added and 2 h later the medium was removed and the cells hydrolyzed with 0.1 N KOH for 30 min. The hydrolysate was saponified with 6% methanolic KOH for 30 min at 80°C. The nonsaponifiable lipids were extracted with light petroleum hydrocarbon and fractionated by TLC using CH_2Cl_2 as developing solvent. The chromatographic plates were analyzed with a Packard (Meriden, CT) radioscanner system 200, and the counts associated with each lipid fraction were measured and expressed as percent of total counts (54).

Time-dependent inactivation: general procedure. Time-dependent inhibition was determined at 37°C by adding the inhibitors to the enzymatic solution of partially purified OSC of pig liver in the absence of substrate. Residual activity was determined under the same conditions as previously described, at 45°C for 30 min after lowering the inhibitor concentration 40 fold; aliquots of 25 μL were withdrawn at time intervals from 30 s to 60 min and transferred to test tubes containing the labeled OS (25 μM) and Tween-80 (0.5 mg/mL) in 950 μL of phosphate buffer. The concentrations of inhibitors obtained after dilution did not inhibit OSC without preincubation.

LD_{50} assay. Female Balb/c mice, one-month-old, were purchased from Charles River Italia (Milano, Italy). They were acclimatized for one week before the start of the experiment.

Increasing amounts of 2-aza-2,3-dihydrosqualene **21**, its HCl salt, 2-aza-2,3-dihydrosqualene alcohol **26** and 2-aza-2,3-dihydrosqualene epoxide **24** were intraperitoneally, intravenously and per os injected into groups of five animals, and the experiments were repeated two or three times. The LD_{50} was calculated from the number of dead mice after seven days. A control group was always set up by injecting the animals with an identical amount of the solution used to prepare the samples: phosphate buffer 0.1 M, pH 7.2, with methanol 25% (vol/vol). At the end of the experiment, the mice were autopsied to assess tissue damage (55).

Pharmacokinetic and biodistribution studies on 2-aza-2,3-dihydrosqualene (HCl salt). The compound was previously labeled with ^{3}H by total synthesis to a specific activity of 1.1×10^{-4} mCi/mol. The labeled compound was used as tracer to monitor the circulatory clearance of the tested drug.

The mice (22 g) were intravenously treated with 2.5 μCi of the tracer. The test doses, including the mass of radiolabeled compound, were 1.5 or 761.5 μg of 2-aza-2,3-dihydrosqualene (HCl salt). The samples were diluted, as described above, to a final volume of 100 μL. Blood samples of 100 μL were drawn from the right retroorbital plexus of groups of five animals at various time points (0, 1, 3, 5, 6, 7, 8, 10, 15, 20 and 30 min, and 1 and 24 h). Capillary Pasteur and Eppendorf vials used for sample collection were washed

in a heparin solution the day before use. Because the animals underwent no more than four drawings, the different groups were overlapped to sample the chosen time range. The blood samples were centrifuged at 10,000 rpm for 5 min (J2-21 Beckman). The plasma was fractionated in 20-μL (amount usually recovered from 50 μL of blood) samples and directly counted in a β-counter (LS 5000 TD Beckman). The radioactivity measured was correlated to the total blood volume, which was assumed to be 10% of body weight.

The pharmacokinetic parameters and slopes were derived using a least-square regression algorithm with a weighting function $1/(y + \bar{y})^2$ (Sigma Plot 4.0 software; Jandel, Erkrath, Germany). Each point of the slope was the mean of seven drawings and is represented as the concentration in μg/mL plotted against time expressed in minutes. The values were obtained adopting a one-compartment phamacokinetic model: $C = C_0^{-kt}$. The plasma samples not used in this experiment were frozen at −80°C for further use in the experiments described below.

Other groups of five animals were similarly injected; the principal organs (liver, kidney, diaphragm, brain, muscle, spleen hearth, and gastrointestinal tract) were removed at different times (0, 3, 5, 7, 10, 15, 20 and 30 min, and 1 and 24 h). The organs were washed twice in physiological solution, wiped, and weighted; amounts of 10–100 mg were prepared and treated with 1 mL of NCS (tissue solubilizer; Amersham, Buckinghamshire, United Kingdom) by stirring overnight at 50°C. Thereafter, and upon addition of acetic acid, 0.034% with respect to the solubilizer volume, the samples were β-counted. The results were plotted as histograms and recorded as radioactivity per organ vs. time expressed in minutes. Mean values for five animals are shown in the graphs (55).

High-performance liquid chromatography (HPLC) and TLC on plasma samples. A Lichrosfer RP 60 Select B (Merck, Darmstadt, Germany) (5 μm) was used with an HPLC Merck Hitachi LC 6000 instrument. Absorbance was monitored at 220 nm. Methanol, (96%), MilliQ water (3%), and isopropylamine (1%) were used as eluent. The samples drawn at different timepoints were diluted with methanol/water (50:50, vol/vol), and peaks were identified by comparison with reference compounds. The eluted fractions were successively chromatographed by TLC (Kieselgel 60 F254, 0.2 mm), using methanol (99.8%) and isopropylamine (0.2%) as solvent. The plates were then radiomonitored with a radioscanner (System 200 Imaging, Canberra Packard). Another set of plasma samples was treated as follows to extract the lipophilic components. Aliquots of 100 μL of plasma were stirred in 200 μL of 12% methanolic KOH and 700 μL 0.1 M phosphate buffer, pH 7.2, at 80°C for 30 min. Lipids were extracted with 3 × 1 mL of light petroleum hydrocarbon and then, after evaporation under vacuum, resuspended in methanol prior to TLC. TLC slabs were eluted as described above.

To monitor the drug biodistribution in different blood components, red cells were precipitated and separately β-counted after solubilization. The plasma was treated with trichloroacetic acid (50% wt/vol) at 4°C for 5 min. After centrifugation at 4000 rpm for 10 min, the pellet of plasma protein was separated, and the samples were extracted as described above. The lipophilic fraction was fractionated by TLC (55).

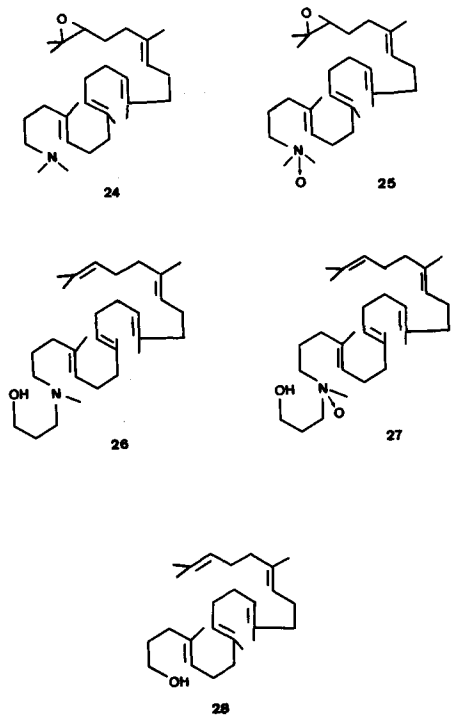
Metabolic studies on urine and excrements. Mice were maintained in metabolic cages (Jencon) with water feeding for 24 h. For urine drawings after 24 h, one mouse per cage was used. Otherwise two mice were necessary to obtain sufficient amounts of sample. Urine was sampled for the following time periods: 0–2, 2–4, 4–7, 7–24, and 0–24 h. Feces was collected 24 h after injection to obtain suitable amounts.

Urine samples after dilution with water (1:1, vol/vol) and filtration (0.2 μm Millipore; Watford, Hertfordshire, United Kingdom) were adjusted to pH 5 with 0.1 M acetate buffer and then stirred at 37°C for 24 h in the presence of glucuronidase sulfatase (1000 U/mL of urine; Boehringer, Mannheim, Germany). The samples were then centrifuged 2000 rpm for 15 min, and the pH was adjusted to 8 with 3 N NaOH. Lipids were extracted with ethyl acetate, and the solvent was evaporated prior to HPLC or TLC analysis. Feces were treated as described (55). Briefly, aliquots of 100 mg were taken up in water, homogenized, and the supernatants centrifuged at 12,000 rpm for 30 min (1-5 50B Beckman). After filtration (0.2 μm), the supernatants were diluted with methanol and extracted prior to TLC (55).

RESULTS AND DISCUSSION

Acyclic azasqualenes. Historically, the first acyclic azasqualene, synthesized in our laboratory, was 2-aza-2,3-dihydrosqualene **21** (Scheme 4) and a series of derivatives, which mimicked the first C-2 carbonium ion or transition state arising from the protonation of the epoxide ring (15,21,24,25,51, 56). These compounds were potent inhibitors of OSC from animals, yeasts, and higher plants, as well as being inhibitors of squalene epoxidase, an enzyme that transforms squalene to OS (6). Thus, the azasqualene derivatives, such as the model compound 2-aza-2,3-dihydrosqualene **21**, did not act specifically on the target enzyme OSC. Moreover, they also inhibited the different OSC of animals, fungi, and higher plants.

We recently found that the introduction of a polar group (epoxy, hydroxy) helped to suppress inhibitory activity on squalene epoxidase without impairing inhibitory potency toward OSC (54) (Scheme 5). By comparing the activity of 2-aza-2,3-dihydrosqualene **21**, the 22,23-epoxy derivative **24**, the corresponding *N*-oxide **25**, azasqualene alcohol **26**, the *N*-oxide derivative **27**, and squalene alcohol **28** on squalene epoxidase from rat liver microsomes, we found that at a concentration of 10 μM, the aza derivatives bearing an epoxy or hydroxy functionality were poor inhibitors (Table 1). In 3T3 fibroblasts, at the lowest concentration (1 μM), azasqualene alcohol **26** and its *N*-oxide derivative **27** were found to be, together with azasqualene **21**, the most active compounds as shown by the suppression of cholesterol biosynthesis by 74 and 90%, respectively (Fig. 1). Moreover, the two compounds, **26** and **27**, proved to be the most specific inhibitors of OSC, as they did not affect squalene epoxidase. We suggest that the



SCHEME 5

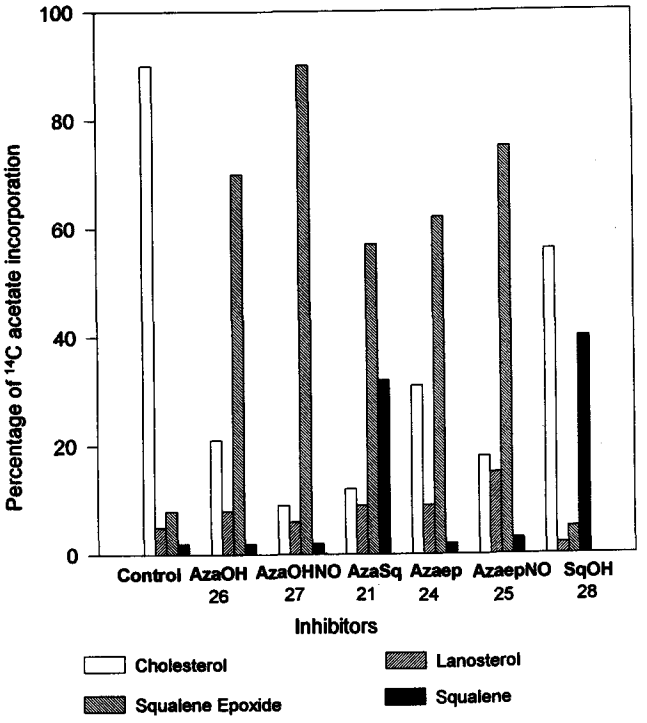


FIG. 1. Incorporation of [^{14}C]acetate into nonsaponifiable lipids by various squalene derivatives at 1 μM concentration. AzaOH, azasqualene alcohol; AzaOHNO, azasqualene alcohol N-oxide; AzaSq, 2-aza-2,3-dihydrosqualene; Azaep, 22,23-epoxy-2-aza-2,3-dihydrosqualene; AzaepNO, 22,23-epoxy-2-aza-2,3-dihydrosqualene N-oxide; SqOH, squalene alcohol.

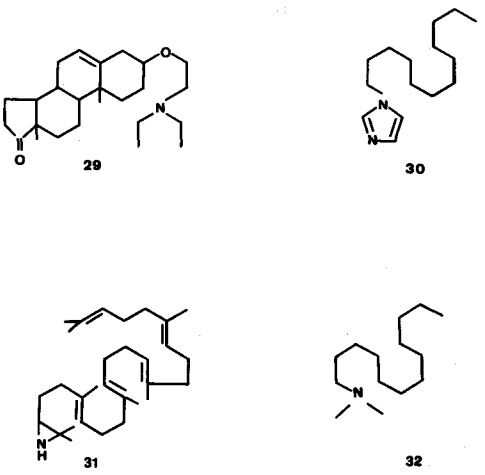
introduction in the structure of 2-aza-2,3-dihydrosqualene **21** of a polar function, such as 3-hydroxypropyl or epoxy, may impair hydrophobic interactions at the active site of squalene epoxidase. On the other hand, the 22,23-epoxyazasqualene derivatives **24** and **25** were as effective as the azasqualene model **21** on OSC from *S. cerevisiae* and *C. albicans* or on cycloartenol and β-amyrin OSC from maize and peas.

Very different compounds, such as U-18666A **29**, *N*-dodecylimidazole **30**, 2,3-iminosqualene **31**, and *N,N*-dimethyldodecylamine **32**, are equally active against higher plant, animal, and yeast OSC (Scheme 6) (15,39,56). Consequently, we suggest that the inhibitors that are able to mimic the high-energy carbonium ion arising from the opening of the epoxidic ring of OS do not strictly need a geometrically correct folding of the squalene skeleton.

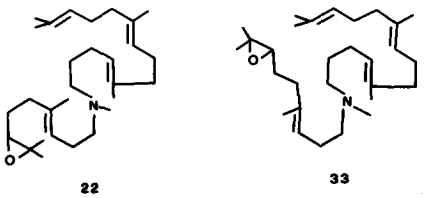
The subsequent step in our strategy was to mimic the C-8 carbonium ion, which is transiently formed during OS cyclization, by synthesizing the two geometrical isomers (*6E*) **22** and (*6Z*)-10-aza-10,11-dihydrosqualene-2,3-epoxide **33** (52) (Scheme 7). As expected, only isomer (*6E*), the carbonium ion analogue corresponding to the natural all *E* OS, was active in all biological systems tested: the IC_{50} values varied from 3–5 μM in yeast (*S. cerevisiae* and *C. albicans*) to 5 μM in animal OSC (Table 2). Moreover, both isomers *E* and *Z*, as opposed to 2-aza-2,3-dihydrosqualene **21**, were inactive against squalene epoxidase from rat liver at the higher concentrations tested.

TABLE 1
Inhibition of Squalene Epoxidase in Rat Liver Microsomes

Inhibitor	Concentration (μM)	Inhibition (%)
Azasqualene (**21**)	10	70
Azasqualene (**24**)	10	8
Azasqualene epoxide *N*-oxide (**25**)	10	14
Azasqualene alcohol (**26**)	10	2
Azasqualene alcohol *N*-oxide (**27**)	10	13
*Tris*norsqualene alcohol (**28**)	10	40



SCHEME 6



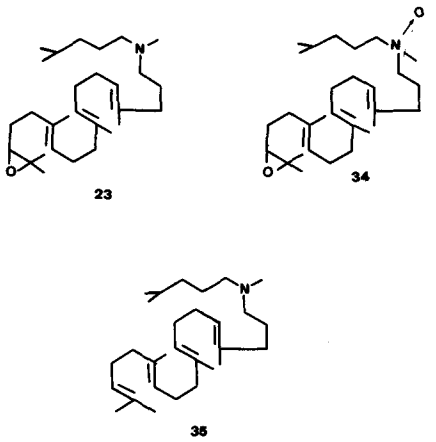
22 33

SCHEME 7

The *in vivo* results obtained by studying the activity of the two isomers in 3T3 fibroblast cell cultures confirmed the selectivity of (*6E*)-10-azasqualene-2,3-epoxide **22** (Viola, F., unpublished data). Indeed, by comparing the incorporation of radioactivity from [2-^{14}C]acetate into the different sterol fractions after preincubation in the presence or absence of inhibitors, it was observed that there was a selective inhibition of OSC by isomer (*6E*), whereas isomer (*6Z*) was practically inactive. The inhibition of cholesterol was dose-dependent: at 0.5 μM it was 60% of the control and at 2 μM the biosynthesis of cholesterol was totally inhibited. At the same time, a correspondent accumulation of OS and 2,3;22,23-dioxidosqualene was observed. Similarly, in the nonsaponifiable lipid fractions from *S. cerevisiae* (57), isomer (*6E*) at 10 μM totally inhibited cholesterol biosynthesis, causing a corresponding dose-dependent accumulation of radioactivity in OS (87.5% of the total label). The main effect of the (*6Z*) isomer was a significant accumulation of the label in the fraction comigrating in TLC with lanosterol. In contrast, (*6E*)-10-azasqualene-2,3-epoxide **22** failed to inhibit OSC from maize, whereas the azadecaline and the 2-aza-2,3-dihydrosqualene derivatives proved to be potent inhibitors (Rahier, A., personal communication).

Thus, the noncyclic inhibitors, that are analogues of the C-8 HEI, must possess the *all-trans* squalenoid geometry of the natural substrate. Moreover, the pro C-8 HEI inhibitor **22** appeared to be more selective than the C-2 HEI azasqualene-type inhibitors such as **21**, as it was able to inhibit OSC from animals and yeasts, whereas it did not inhibit higher plant OSC (Rahier, A., personal communication).

The next step in our program was the synthesis of acyclic azasqualenes mimicking the C-20 carbonium ion intermediates, such as 19-aza-18,19,22,23-tetrahydrosqualene-2,3-epoxide **23**, its *N*-oxide **34**, and 19-aza-18,19,22,23-tetrahydrosqualene **35** (58) (Scheme 8). We assayed the inhibition caused by these compounds *in vitro* on partially purified OSC from pig liver, and on OSC associated with microsomes from rat and pig liver, and from *S. cerevisiae* and *C. albicans*.



23 34 35

SCHEME 8

The most active compounds, 19-azasqualene-2,3-epoxide **23** and its *N*-oxide **34** showed *in vitro* an inhibitory activity on animal OSC similar to that found for the parent compound 2-aza-2,3-dihydrosqualene **21** and the corresponding *N*-oxide (Table 3). The presence of an epoxide ring is essential for activity, as 19-azasqualene **35** was not active under the same conditions. The two 19-azasqualene-2,3-epoxide derivatives **23** and **34** were not effective inhibitors of OSC from yeast microsomes or from higher plants, such as cycloartenol OSC or β-amyrin OSC (Rahier, A., personal communication), and they did not inhibit animal squalene epoxidase.

In Hep G2 hepatoma cultures, incubation with [2-^{14}C]-acetate in the presence of inhibitor **23** caused a decrease in the radioactivity in the cholesterol fraction (IC_{50} = 0.2 μM), whereas an accumulation of the label in the fractions corresponding to OS and 2,3;22,23-dioxidosqualene was observed. Consequently, 19-azasqualene-2,3-epoxide **23** is a much more

TABLE 2
IC_{50} Values (μM) of Inhibition of Microsomal 2,3-Oxidosqualene Cyclase by (6*E*-22) and (6*Z*)-10-Azasqualene-2,3-epoxide (33)[a]

Microsomes	Isomer *E*	Isomer *Z*
Rat liver[b]	4.8	>20
Pig liver[b]	5	ND
Saccharomyces cerevisiae[c]	5	>100
Candida albicans[d]	3	>100

[a]The values are means of two different experiments; ND, not determined; IC_{50}, the concentration of inhibitor which reduces the enzymatic conversion by 50%.
[b]Microsomal protein concentration was 1 mg/mL for pig liver and 5 mg/mL for rat liver.
[c]Microsomal protein concentration was 2 mg/mL.
[d]Microsomal protein concentration was 3 mg/mL.

TABLE 3
IC_{50} Values (μM) of Inhibition of 2,3-Oxidosqualene Cyclase by 19-Azasqualene-2,3-epoxide (23) and 19-Azasqualene-2,3-epoxide *N*-oxide (34)[a]

	Compounds	
Enzymatic assay[a]	**23**	**35**
Partially purified pig enzyme	1.7	7
Rat liver[b]	7.5	ND
Pig liver[b]	1.5	ND
Saccharomyces cerevisiae[c]	35	100
Candida albicans[c]	22	55

[a]The values are means of two different experiments. Abbreviations as in Table 2.
[b]Microsomal protein concentration was 1 mg/mL for pig liver and 5 mg/mL for rat liver.
[c]Microsomal protein concentration was 3 mg/mL.

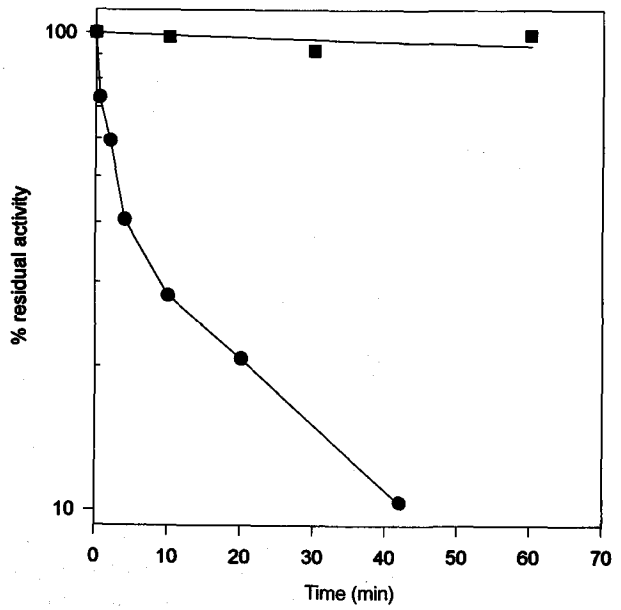


FIG. 2. Time-dependent inhibition of 2,3-oxidosqualene cyclase (OSC) by 19-azasqualene-2,3-epoxide **23** (5 μM) (●) and 19-azasqualene **35** (50 μM) (■). OSC was preincubated at 37°C in phosphate buffer in the presence of the inhibitors. At intervals, 25 μL were withdrawn and diluted to 1 mL of phosphate buffer containing the substrate 2,3-oxidosqualene. The enzymatic activity was determined as % of the activity obtained after preincubation in the absence of inhibitors.

selective inhibitor than the corresponding 10-azasqualene-2,3-epoxide **22** and 2-aza-2,3-dihydrosqualene **21**, because it inhibited mainly mammalian OSC rather than yeast or higher plant OSC.

Surprisingly, 19-azasqualene-2,3-epoxide **23** was also able to inactivate OSC from pig liver in a time-dependent manner (Viola, F., unpublished data); the inactivation was very effective. The inhibitor, at a concentration of 10 μM, caused the loss of 50% of the enzymatic activity after 1 min of preincubation (Fig. 2). By contrast, 19-azasqualene **35** was ineffective at the higher concentrations tested (50 μM). When the enzyme, preincubated with compound **23**, was passed after the preincubation through a membrane anion exchange cartridge, the activity was not restored.

We could speculate that 19-azasqualene-2,3-epoxide **23**, in order to inhibit OSC, must be first recognized by the enzyme through the epoxide ring and then correctly folded or cyclized to obtain the conformation that best mimics the C-20 carbonium ion. At this point, this postulated intermediate could covalently link to the enzyme, acting as a site-directed inhibitor, or it could be strictly associated with the enzyme as a tight-binding inhibitor. In order to test this, we assayed (*6E*)-10-aza-10,11-dihydrosqualene-2,3-epoxide **22** as a time-dependent inhibitor. We found that **22** was also a pseudo-irreversible inhibitor of OSC, as it was able to inhibit 50% of the enzymatic activity after a few minutes of preincubation, whereas isomer (*6Z*) **33** was found to be inactive. Therefore, it appears that the azasqualenes with the correct all *E* geometry, bearing an epoxide terminal ring and a nitrogen corresponding to the C-8 or C-20 intermediates, act as irreversible inhibitors.

Thus, we can make the following conclusions in regard to the azasqualene derivatives: (i) All the azasqualenes designed could mimic the corresponding HEI formed by cyclization of OS, as shown by their good inhibitory activity. This can be considered indirect evidence of the known isoprenic rule which postulates the formation of a series of conformationally rigid carbonium ion intermediates (from C-2 to C-20). (ii) A progressive selectivity was shown in regard to the inhibition of OSC going from C-2 to pro C-8 and pro C-20 HEI azasqualene analogues. This could also mean that the OSC active site complementary to the C-8 and C-20 HEI differs considerably in mammalian, yeast, and higher plant enzymes. (iii) The inhibitory efficacy of azasqualenes appears to be related to their relative conformational flexibility, which allows these compounds to bind to the OSC in their ground state and then trigger a rapid conformational change at the active site of the enzyme. This step seems necessary to obtain a strict interaction with the cyclic carbonium ion formed during the cyclization of OS.

Initial pre-clinical studies with 2-aza-2,3-dihydrosqualene. We initially compared the toxicity of different azasqualene derivatives in mice (Table 4) upon administering different amounts of the dugs by the intravenous, the intraperitoneal, and the oral route (55).

The LD_{50} of 2-aza-2,3-dihydrosqualene **21** and of its HCl salt following intravenous injection was calculated to be 38 mg/kg mouse; with up to 34 mg/kg no pathological lesions were observed. At the highest dose (60 mg/kg mouse), death of the animals occurred quickly with widespread hemorrhage of intestine, kidney, spleen, and lung. After intraperitoneal injection, the LD_{50} of the **21** HCl salt was 80 mg/kg mouse; the LD_{50} after per os administration was approximately 800 mg/kg. 2-Aza-2,3-dihydrosqualene-22,23-epoxide **24** proved to be nontoxic; after intraperitoneal administration of the drug, death did not occur up to doses of 2 g/kg mouse.

Pharmacokinetic analysis of the **21** HCl salt upon intravenous injection revealed that the plasma disappearance could be described by a monoexponential function with a mean half-life of 5.62 min (Fig. 3). The distribution of the drug indicated that after 30 min, most of HCl salt of com-

TABLE 4
***In vivo* Toxicity[a]**

Compound	Administered	Dose	LD_{50}
21	iv	31; 38; 40; 60	38
21 (HCl salt)	iv	31; 38; 40; 60	38
26	iv	33; 60; 80	n.t.
21 (HCl salt)	ip	34; 80; 120; 220	80
24	ip	93; 120; 140; 200; 400; 1200; 2000	n.t.
26	ip	40; 120; 200; 320; 600	n.t.
21 (HCl salt)	po	40; 150; 200; 400; 800	800

[a]Concentrations are expressed in mg/kg mouse. iv, intravenous; n.t., not toxic; ip, intraperitoneally; po, per os; LD_{50}, standard deviations were less than 2.9%, and are not reported.

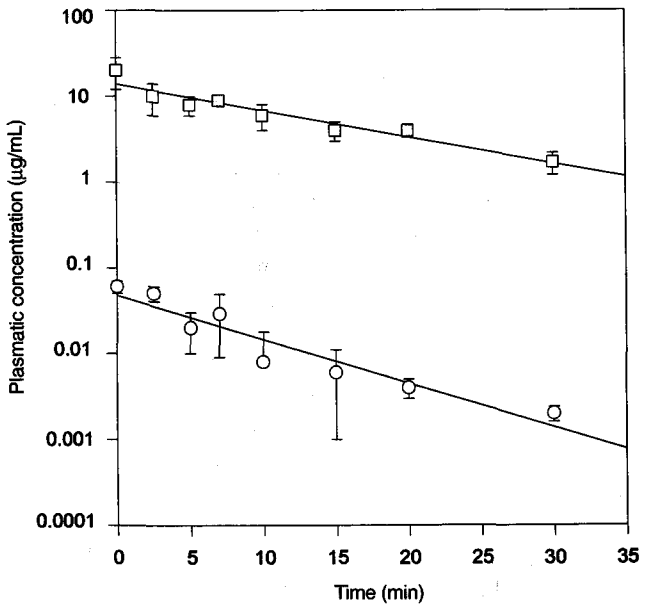


FIG. 3. Plasma clearance: Balb/c mice were injected intravenously with radiolabeled 2-aza-2,3-dihydrosqualene **21** (HCl salt) at different doses. Geometric mean (points) and SD (bars) of results, obtained in seven drawings, are reported; ○, 1.5 µg; □, 761.5 µg.

pound **21** was present in the liver and in the gut, while the remainder (less than 10%) was in the plasma and in the other tissues (Fig. 4).

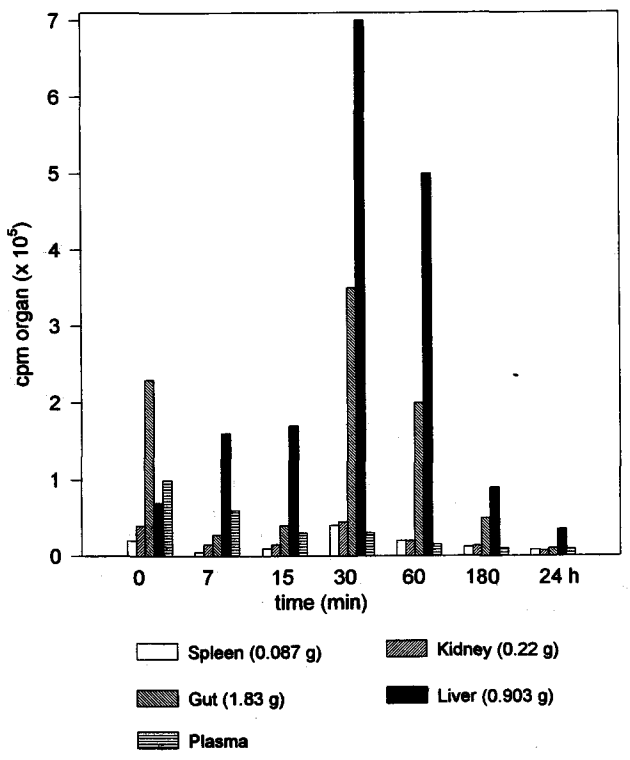


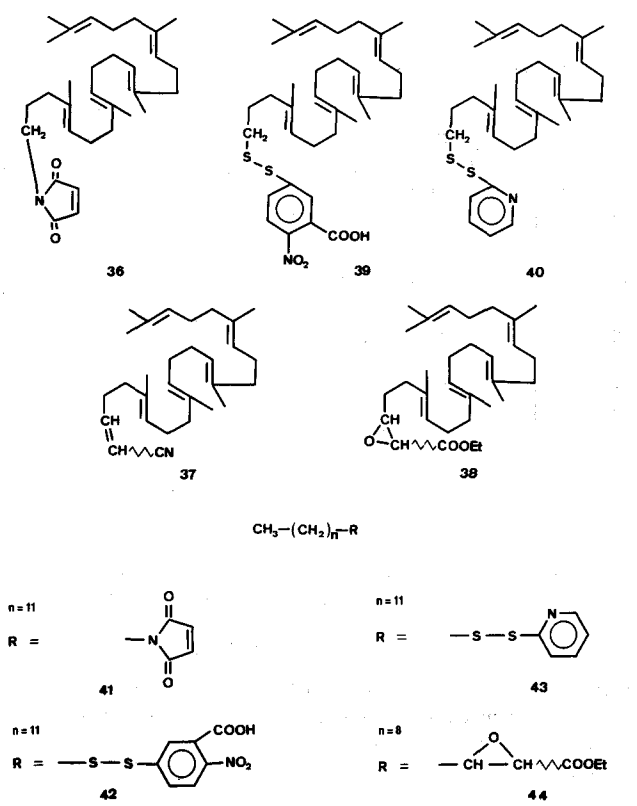
FIG. 4. Biodistribution of 2-aza-2,3-dihydrosqualene **21** (HCl salt) in Balb/c mice; means of five animals are shown. SD values were less than ±10%.

Thus, 2-aza-2,3-dihydrosqualene **21** HCl salt was characterized by a short plasma half-life, a high apparent volume of distribution, and an elevated total clearance. Moreover, this compound was mainly concentrated in the liver and in the gut, and also associated, to a significant extent, with red blood cells and plasma proteins. Plasma, urine, and feces showed the presence of a single metabolite identified as 2-aza-2,3-dihydrosqualene *N*-oxide, previously found (59) after incubation of **21** with rat liver microsomes. This compound has shown high cholesterol-inhibiting activity in animal cells (60). The observed metabolism of **21** may contribute to the hepatic elimination of the parent drug, and eventually also from the enterohepatic circulation. The high concentration of the **21** HCl salt in liver and gut, its recovery in feces accompanied by its *N*-oxide metabolite, and the low concentration of the drug in extrahepatic tissues suggest hepatic extraction and enterohepatic shunt.

According to this view, lovastatin, simvastatin, and pravastatin, which often are the drugs of choice in the treatment of familiar hypercholesterolemia (61–64), appear pharmacokinetically related to 2-aza-2,3-dihydrosqualene **21**.

Squalene derivatives as thiol-modifying agents. In order to obtain further insight into the mechanism of OS cyclization in different OSC enzymes, a series of squalene derivatives was designed that could covalently link to an active-site thiol group.

The following types of potential irreversible inhibitors of



SCHEME 9

TABLE 5
Inhibition of Pig Liver 2,3-Oxidosqualene Cyclase by Squalene and Dodecyl Derivatives[a]

Compounds	Percent inhibition
36	82
37	25
38	37
39	65
40	67
41	83
42	73
43	67
44	0

[a]The concentration of the inhibitors was 200 μM. Values are the means of two independent determinations which differed less than 5%.

OSC carrying a squalenoid or dodecyl skeleton (Scheme 9) were synthesized (65): (i) Michael-type inhibitors such as squalene maleimide **36** and the α,β-unsaturated nitrile **37**; (ii) Calpain-type (66) oxirane inhibitors such as **38**: in some proteases these compounds would be attacked after protonation of the epoxide group by a suitable nucleophilic thiol group of the enzyme; (iii) Ellman-type squalenoid inhibitors such as the 2-nitro-5-dithiobenzoic derivative **39** (squalene Ellman) and the 2-dithiopyridyl derivative **40**.

By comparing the activity of the squalene **36–40** and dodecyl **41–44** derivatives, we found that the maleimido derivatives **36** and **41** were the most active inhibitors of mammalian OSC, followed by the Ellman-type compounds **39** and **42**. THe α,β-unsaturated nitrile **37** and the oxirane derivatives **38** and **44** showed little or no activity (Table 5). By evaluating the time-dependent inactivation of these compounds following their incubation with the mammalian enzyme, we found that only the maleimide derivatives were effective as irreversible inhibitors. In particular, we found that the substrate OS was not able to protect the enzyme from inactivation by *N*-dodecylmaleimide, but partially protected the enzyme after incubation for up to 90 min with 50 μM of squalene maleimide **36** (Fig. 5). The degree of substrate protection was statistically significant (15–25%) and was also obtained at 250 and 500 μM concentration of the inhibitor. Similar results had previously been obtained by Schuber. The competitive inhibitor 20-oxa-22,23-dihydrosqualene-2,3-epoxide (41) was not able to protect the enzyme by *N*-ethylmaleimide inactivation (47).

Surprisingly, the squalene Ellman derivative **39** (3-carboxy-4-nitrophenyldithio-1,1′,2-*tris*-norsqualene; CNDT-squalene) did not behave as a time-dependent irreversible inhibitor of liver OSC. In contrast, this compound was found to be an irreversible inhibitor of OSC from *S. cerevisiae* with an apparent binding constant K_i = 1.21 mM and an inactivation rate constant at saturation $k_{app.max.}$ = 0.220 min^{-1} (67).

In Figure 6 is shown the time-dependent inactivation of yeast OSC after pre-incubation with CNDT-squalene **39** at

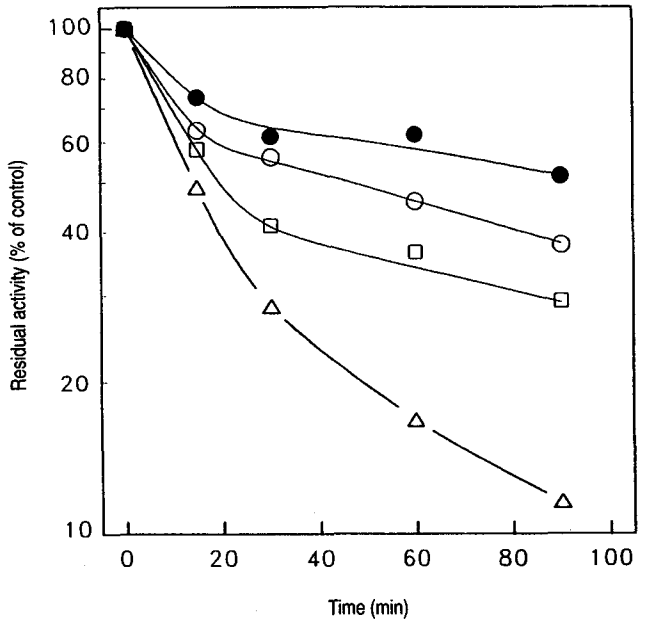


FIG. 5. Time-dependent inhibition of 2,3-oxidosqualene cyclase (OSC) with different concentrations of squalene maleimide **36**. Residual activity (expressed as % of controls without inhibitor) was determined after preincubation of solubilized OSC for increasing time periods (15–90 min) with squalene maleimide 50 μM (○), 100 μM (□), and 200 μM (△). The black circles indicate the residual activity after preincubation of 50 μM inhibitor in the presence of 500 μM of 2,3-oxidosqualene.

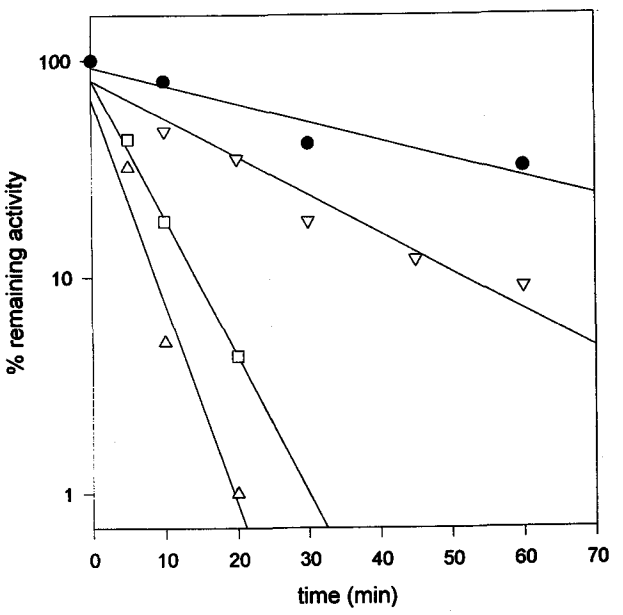


FIG. 6. Time-dependent activation of yeast 2,3-oxidosqualene cyclase by 3-carboxy-4-nitrophenyldithio-1,1′-2-*tris*-norsqualene **39**. The enzyme solubilized from microsomes with Nonidet P-40 (Sigma, Milano, Italy) was incubated (0.3 mg protein/mL, 0.1 M K/Na phosphate buffer, pH 7.1, containing 1 mg/mL Nonidet P-40) at 35°C with 0.0 (●), 0.1 (▽), 0.5 (□), and 1 mM (△) reagent. At time intervals, 50-μL aliquots were withdrawn and diluted with 450 μL 0.1 M K/Na phosphate buffer, pH 7.1, containing 0.5 mg/mL Nonidet P-40, 0.2 mg/mL Tween 80, [^{3}H]2,3-oxidosqualene (50,000 dpm) and 2,3-oxidosqualene (25 μM). The mixture was then incubated for 30 min at 35°C to determine the residual activity, which is expressed as % of control (no reagent).

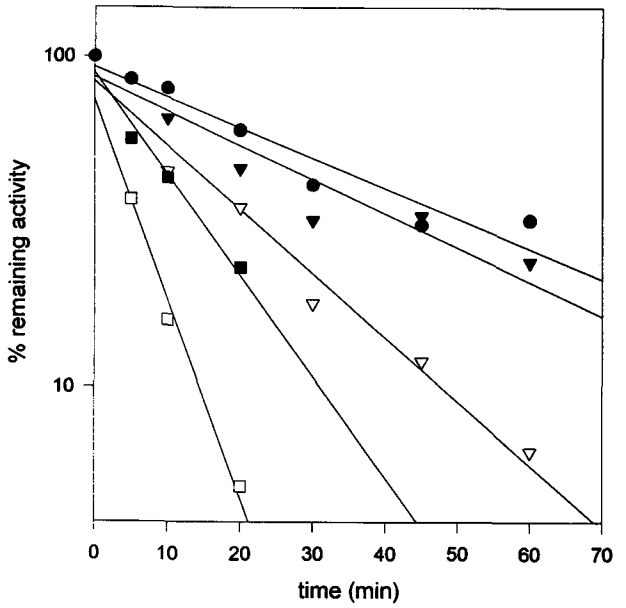


FIG. 7. Protection by 2,3-oxidosqualene (substrate) against 3-carboxy-4-nitrophenyldithio-1,1′-2-*tris*-norsqualene (CNDT)-squalene **39** inactivation of yeast 2,3-oxidosqualene cyclase. Experiments were carried out as described in the legend to Figure 6. CNDT-squalene concentrations were 0.0 (●), 0.1 (▼), and 0.5 mM (■) in experiments with substrate (0.5 mM) as a protecting agent, and 0.1 (▽) and 0.5 mM (□) in experiments with no substrate present.

different concentrations (0.1, 0.5, and 1 mM). The inactivation was reversed by tenfold dilution of the enzyme or by direct reduction of the thiol reagent with dithiothreitol or mercaptoethanol (both ineffective on OSC). In order to localize the thiol groups involved in the inactivation of OSC, substrate protection experiments were performed in the presence of inactivator. Substrate, at a saturating concentration, clearly protected the yeast OSC against inactivation (Fig. 7).

By contrast, the Ellman's reagent 5,5′-dithio-*bis*-(2-nitrobenzoic) acid (DTNB) (68) proved to be effective as a time-dependent inactivator, but failed to be affected by the presence of the substrate. These results were confirmed by following photometrically the release of TNB (thionitrobenzoate) from CNDT-squalene or DTNB in the presence or in the absence of the substrate. While the release of TNB from DTNB was not affected by saturating concentrations of the substrate, there was a significant reduction of TNB release from CNDT-squalene **39** under the same conditions. Replacement of the squalene moiety by a dodecyl residue also made the inhibition less specific and less active. The squalene group of **39** seems to act as a carrier able to lead the modifying agent into the active site of the enzyme.

Much more work will be necessary to arrive at a definitive view on the role of the single amino acid residues in the overall enzymatic catalysis leading to the cyclization of squalene 2,3-epoxide to tetracyclic or pentacyclic triterpenes. At the moment, on the basis of our experiments, we can postulate the existence of a set of thiol groups outside and inside the OSC active site (in animals and yeasts). The latter can potentially interfere with each step of OS transformation, namely, the initial protonation, the cyclization, the following rearrangement, and the final abstraction of a proton leading to the final product.

In conclusion, our strategy of designing reversible HEI analogue inhibitors (acyclic azasqualene derivatives) or irreversible squalene-derived thiol reagents, as squalene maleimide or squalene Ellman derivatives, has been useful in obtaining further insights into the mechanism of OS cyclization as well as the design of specific hypocholesterolemic or antifungal agents.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministero della Ricerca Scientifica e Tecnologica and from CNR, Progetto Finalizzato Chimica Fine. Thanks are also due to Dr. P. Milla for her helpful suggestions and to D. Zonari for excellent technical assistance.

REFERENCES

1. Corey, E.J., Russey, W.E., and Ortiz de Montellano, P.R. (1966) *J. Am. Chem. Soc. 88*, 4750–4751.
2. van Tamelen, E.E., Willett, J.D., Clayton, R.B., and Lord, K.E. (1966) *J. Am. Chem. Soc. 88*, 4752–4754.
3. Yamamoto, S., Lin, K., and Bloch, K. (1969) *Proc. Natl. Acad. Sci. USA 63*, 110–117.
4. van Tamelen, E.E. (1982) *J. Am. Chem. Soc. 104*, 6480–6481.
5. Nes, W.D. (1990) *Recent Adv. Phytochem. 24*, 283–327.
6. Cattel, L., and Ceruti, M. (1992) in *Physiology and Biochemistry of Sterols* (Patterson, G.W., and Nes, W.D., eds.) pp. 50–82, American Oil Chemists' Society, Champaign.
7. Abe, I., Rohmer, M., and Prestwich, G.D. (1993) *Chem. Rev. 93*, 2189–2206.
8. Goodwin, T.W. (1980) in *Biosynthesis of Isoprenoid Compounds* (Porter, J.W., and Spurgeon, S.L., eds.) Vol. 1, pp. 443–480, Wiley, New York.
9. Rahier, A., Taton, M., and Benveniste, P. (1990) in *Biochemistry of Cell Walls and Membranes in Fungi* (Kuhn, P.J., Trinci, A.P.J., Jung, M.J., Goosey, M.W., and Copping, L.G., eds.) pp. 205–222, Springer-Verlag, Berlin.
10. Rahier, A., Taton, M., Bouvier-Navé, P., Schmitt, P., Benveniste, P., Schuber, F., Narula, A.S., Cattel, L., Anding, C., and Place, P. (1986) *Lipids 21*, 52–62.
11. Eschenmoser, A., Ruzicka, L., Jeger, O., and Arigoni, D. (1955) *Helv. Chim. Acta 38*, 1890–1904.
12. Cornforth, J.W., Cornforth, R.H., Donninger, C., Popjak, G., Shimizu, Y., Ichii, S., Forchielli, E., and Caspi, E. (1965) *J. Am. Chem. Soc. 87*, 3224–3228.
13. van Tamelen, E.E. (1975) *Acc. Chem. Res. 8*, 152–158.
14. van Tamelen, E.E., and James, D.R. (1977) *J. Am. Chem. Soc. 99*, 950–952.
15. Cattel, L., Ceruti, M., Viola, F., Delprino, L., Balliano, G., Duriatti, A., and Bovier-Navé, P. (1986) *Lipids 21*, 31–38.
16. Johnson, W.S., Telfer, S.J., Cheng, S., and Schubert, U. (1987) *J. Am. Chem. Soc. 109*, 2517–2518.
17. Corey, E.J., and Virgil, S.C. (1991) *J. Am. Chem. Soc. 113*, 4025–4026.
18. Corey, E.J., Virgil, S.C., and Sarshar, S. (1991) *J. Am. Chem. Soc. 113*, 8171–8172.
19. Bohlmann, R. (1992) *Angew. Chem. Int. Ed. Engl. 31*, 582–584.
20. Rahier, A., Taton, M., and Benveniste, P. (1990) *Biochem. Soc. Trans. 18*, 48–52.

21. Cattel, L., Ceruti, M., Balliano, G., Viola, F., Grosa, G., and Schuber, F. (1989) *Steroids 53*, 363–391.
22. Goldstein, J.L., and Brown, M.S. (1990) *Nature 343*, 425–430.
23. Rahier, A., Narula, A.S., Benveniste, P., and Schmitt, P. (1980) *Biochem. Biophys. Res. Commun. 92*, 20–25.
24. Delprino, L., Balliano, G., Cattel, L., Benveniste, P., and Bouvier, P. (1983) *J. Chem. Soc., Chem. Commun.*, 381–382.
25. Ceruti, M., Balliano, G., Viola, F., Cattel, L., Gerst, N., and Schuber, F. (1987) *Eur. J. Med. Chem. 22*, 199–208.
26. Ceruti, M., Viola, F., Balliano, G., Grosa, G., Caputo, O., Gerst, N., Schuber, F., and Cattel, L. (1988) *Eur. J. Med. Chem. 23*, 533–537.
27. Taton, M., Benveniste, P., and Rahier, A. (1986) *Biochem. Biophys. Res. Commun. 138*, 764–770.
28. Abe, I., Tomesch, J.C., Wattanasin, S., and Prestwich, G.D. (1994) *Nat. Prod. Rep.*, 279–301.
29. Dodd, D.S., and Oehlschlager, A.C. (1992) *J. Org. Chem. 57*, 2794–2803.
30. Dodd, D.S., Oehlschlager, A.C., Georgopapadakou, N.H., Polak, A.-M., and Hartman, P.G. (1992) *J. Org. Chem. 57*, 7226–7234.
31. Taton, M., Benveniste, P., Rahier, A., Johnson, W.S., Liu, H.-t., and Sudhakar, A.R. (1992) *Biochemistry 31*, 7892–7898.
32. Gerst, N., Duriatti, A., Schuber, F., Taton, M., Benveniste, P., and Rahier, A. (1988) *Biochem. Pharmacol. 37*, 1955–1964.
33. Wannamaker, M.W., Waid, P.P., Moore, W.R., Schatzman, G.L., Van Sickle, W.A., and Wilson, P.K. (1993) *Bioorg. Med. Chem. Lett. 3*, 1175–1178.
34. Wannamaker, M.W., Waid, P.P., Van Sickle, W.A., McCarty, J.R., Wilson, P.K., Schatzman, G.L., and Moore, W.R. (1992) *J. Med. Chem. 35*, 3581–3583.
35. Ruhl, K.K., Anzalone, L., Arguropoulos, E.D., Gayen, A.K., and Spencer, T.A. (1989) *Bioorg. Chem. 17*, 108–120.
36. Rahier, A., Taton, M., Schmitt, P., Benveniste, P., Place, P., and Anding, C. (1985) *Phytochemistry 24*, 1223–1232.
37. Taton, M., Benveniste, P., and Rahier, A. (1987) *Phytochemistry 26*, 385–392.
38. Viola, F., Ceruti, M., Balliano, G., Caputo, O., and Cattel, L. (1990) *Il. Farmaco 45*, 965–978.
39. Duriatti, A., Bouvier-Navé, P., Benveniste, P., Schuber, F., Delprino, L., Balliano, G., and Cattel, L. (1985) *Biochem. Pharmacol. 34*, 2765–2777.
40. Ceruti, M., Delprino, L., Cattel, L., Bouvier-Navé, P., Duriatti, A., Schuber, F., and Benveniste, P. (1985) *J. Chem. Soc., Chem. Commun.*, 1054–1055.
41. Ceruti, M., Viola, F., Dosio, F., Cattel, L., Bouvier-Navé, P., and Ugliengo, P. (1988) *J. Chem. Soc., Perkin Trans. 1*, 461–469.
42. Xiao, X.-y., and Prestwich, G.D. (1991) *J. Am. Chem. Soc. 113*, 9673–9674.
43. Abe, I., Bai, M., Xiao, X.-y., and Prestwich, G.D. (1992) *Biochem. Biophys. Res. Commun. 187*, 32–38.
44. Abe, I., and Prestwich, G.D. (1994) *J. Biol. Chem. 269*, 802–804.
45. Beastall, G.H., Rees, H.H., and Goodwin, T.W. (1971) *FEBS Lett. 18*, 175–178.
46. Penefsky, H.S., and Tzagoloff, A. (1971) *Methods Enzymol. 22*, 204–219.
47. Duriatti, A., and Schuber, F. (1988) *Biochem. Biophys. Res. Commun. 151*, 1378–1385.
48. Abe, I., Sankawa, U., and Ebizuka, Y. (1992) *Chem. Pharm. Bull. 40*, 1755–1760.
49. Hoshino, T., Williams, H.J., Chung, Y., and Scott, A.I. (1991) *Tetrahedron 47*, 5925–5932.
50. Seckler, B., and Poralla, K. (1986) *Biochim. Biophys. Acta 881*, 356–363.
51. Balliano, G., Viola, F., Ceruti, M., and Cattel, L. (1988) *Biochim. Biophys. Acta 959*, 9–19.
52. Ceruti, M., Balliano, G., Viola, F., Grosa, G., Rocco, F., and Cattel, L. (1992) *J. Med. Chem. 35*, 3050–3058.
53. Balliano, G., Viola, F., Ceruti, M., and Cattel, L. (1992) *Arch. Biochem. Biophys. 293*, 122–129.
54. Ceruti, M., Viola, F., Balliano, G., Grosa, G., Rocco, F., Biglino, G., and Cattel, L. (1992) *Atti Accad. Sci. Torino 126*, 131–141.
55. Brusa, P., Dosio, F., Ceruti, M., Grosa, G., and Cattel, L. (1993) *Atti Accad. Sci. Torino 127*, 239–256.
56. Cattel, L., Ceruti, M., Balliano, G., and Viola, F. (1992) in *Regulation of Isopentenoid Metabolism*, (Nes, W.D., Parish, E.J., and Trzaskos, J.M., eds.) pp. 174–191, American Chemical Society, Washington, D.C.
57. Balliano, G., Milla, P., Ceruti, M., Viola, F., Carrano, L., and Cattel, L. (1993) *FEBS Lett. 320*, 203–206.
58. Ceruti, M., Rocco, F., Viola, F., Balliano, G., Grosa, G., Dosio, F., and Cattel, L. (1993) *Eur. J. Med. Chem. 28*, 675–682.
59. Viola, F., Grosa, G., Ceruti, M., Caputo, O., and Cattel, L. (1989) *Biochem. Pharmacol. 38*, 2497–2503.
60. Gerst, N., Schuber, F., Viola, F., and Cattel, L. (1986) *Biochem. Pharmacol. 35*, 4243–4250.
61. Hoeg, J.M., and Brewer, Jr., H.B. (1987) *J. Am. Med. Assoc. 258*, 3532–3536.
62. Vickers, S., Duncan, C.A., Chen, I.-W., Rosegay, A., and Duggan, D.E. (1990) *Drug. Metab. Disp. 18*, 138–145.
63. Bocan, T.M.A., Ferguson, E., McNally, W., Uhlendorf, P.D., Bak Mueller, S., Dehart, P., Sliskovic, D.R., Roth, B.D., Krause, B.R., and Newton, R.S. (1992) *Biochim. Biophys. Acta 1123*, 133–144.
64. Duggan, D.E., Chen, I.-W., Bayne, W.F., Halpin, R.A., Duncan, C.A., Schwartz, M.S., Stubbs, R.J., and Vickers, S. (1989) *Drug. Metab. Disp. 17*, 166–173.
65. Grosa, G., Viola, F., Ceruti, M., Brusa, P., Delprino, L., Dosio, F., and Cattel, L. (1994) *Eur. J. Med. Chem. 29*, 17–23.
66. Mehdi, S. (1991) *Trends Biochem. Sci. 16*, 150–153.
67. Balliano, G., Grosa, G., Milla, P., Viola, F., and Cattel, L. (1993) *Lipids 28*, 903–906.
68. Ellman, G.L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

[Received July 22, 1994, and in revised form January 23, 1995; Revision accepted January 25, 1995]

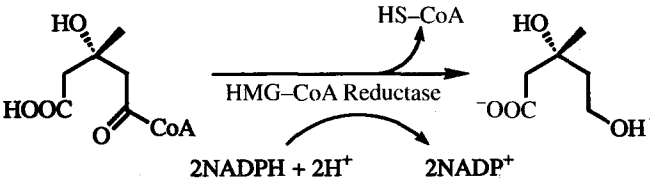
Side-Chain Oxysterol Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity[1]

Edward J. Parish*, Sarawanee C. Parish and Shengrong Li
Department of Chemistry, Auburn University, Auburn, Alabama 36849

ABSTRACT: Side-chain oxysterols are known to be potent inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, a key regulatory enzyme in the biosynthesis of sterols. Structural variations in the side-chain oxysterols influence enzyme inhibition. Under certain conditions, biological systems have been induced to produce side-chain oxysterols, adding support to the hypothesis that oxysterols may be natural regulators of sterol biosynthesis in the intact cell. Specific inhibition of sterol biosynthesis is of interest as it may prove useful in the prevention or reversal of various cardiovascular disease states, as well as in the control of normal and abnormal cell growth.
Lipids 30, 247–251 (1995).

Sterol biosynthesis in mammalian systems has been intensely studied for several decades (1–3). Interest in the cholesterol pathway has increased following clinical observations that the incidence of cardiovascular disease is greater in individuals with levels of serum cholesterol higher than average (4). More recently, the results of numerous clinical studies have indicated that lowering serum cholesterol levels may reduce the risk of coronary heart disease (5) and even promote the regression of atherosclerotic lesions (6,7). Serum cholesterol levels can sometimes be controlled by restricting the dietary intake of cholesterol and fat. However, with a significant number of patients, this approach does not result in the lowering of serum cholesterol concentrations. This makes the development of other approaches to reduce serum cholesterol levels an important goal of cardiovascular research.

A direct approach to this problem has been the development of inhibitors of cholesterol or sterol biosynthesis. A key regulatory enzyme of cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase (EC 1.1.134) which catalyzes the reductive deacylation of HMG–CoA to yield mevalonate (Scheme 1) (8,9). The addition of highly purified cholesterol to cultured cells does not affect the activity of HMG–CoA reductase or the rate of sterol biosynthesis (10,11). However, various oxysterols have been shown to be potent suppressors of HMG–CoA reductase activity resulting in marked reductions of sterol biosynthesis (12). These observations led Kandutsch and co-workers (12,13) to hypothesize that oxysterols, rather than cholesterol, may function as the natural regulators of HMG–CoA reductase activity and sterol biosynthesis.



SCHEME 1

Oxysterols, i.e., sterols bearing a second oxygen function in addition to that at carbon-3, have been demonstrated to possess diverse biological activities (8,14–16). These include cytotoxicity, atherogenicity, carcinogenicity, mutagenicity, hypocholesterolemia, and various effects on certain enzymes. In addition, several oxysterols have been isolated from drugs used in folk medicine for the treatment of cancer (17–19). Other studies have shown that several oxysterols have significant activity in inhibiting DNA synthesis in cultured cells (20,21). Oxysterols having the second oxygen function in the side-chain region of the sterol structure are termed side-chain oxysterols.

An important property of many oxysterols is their ability to inhibit HMG–CoA reductase activity in cultured mammalian cells (8,14,22). This suppression of activity has been found to vary over a wide range depending on the structural features of the oxysterol. As a general trend, inhibitory activity increases as the distance between carbon-3 and the second oxygen group becomes greater. Sterols with an additional oxygen function in ring D and the side chain have been shown to have the highest activity. An intact side chain is a requirement for potent activity; a decrease in the length of the (isooctyl) side chain results in decreased activity (23). Another structure–activity relationship has been noted between inhibitory activity and the extent to which the second oxygen function is sterically hindered. In general, axial hydroxyl groups are more hindered and exert lower activities than the less hindered equatorial conformation (8,23,24). Steric hindrance from other parts of the steroid molecule can result in diminished activity (i.e., effect of carbon-14 alkyl substituents

[1]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed.

Abbreviations: CHO, Chinese hamster ovary; HMG–CoA, 3-hydroxy-3-methylglutaryl CoA.

Copyright © 1995 by AOCS Press

on the carbon-15 hydroxyl group) (25). It has been suggested that oxygen functions in conformationally flexible positions, such as those in ring D and in the side chain, and produces steroids with greater inhibitory activity due to increased effectiveness of hydrogen bonding or hydrophilic interactions with receptor molecules (16).

THE OXYSTEROL REGULATION HYPOTHESIS

The experimental evidence summarized here suggests a regulatory mechanism which, by analogy to steroid hormone receptors and bacterial induction–repression systems, requires a binding protein to recognize oxysterols and mediate subsequent cellular events. There is evidence for the existence of a specific cytosolic receptor protein for oxysterols (23,26). After the activities of a number of sterols were evaluated, a good correlation was found between the actions of certain oxysterols on HMG–CoA reductase in L cells and their affinity for an oxysterol binding protein (23) Moreover, the actions of oxysterols which depress the rate of cholesterol biosynthesis from lanosterol and possibly inhibit the 14-demethylation of lanosterol are also postulated to exert their actions by an oxysterol binding protein (22,27,28).

The side-chain oxysterol most frequently utilized in the study of HMG–CoA reductase regulation is cholest-5-ene-3β,25-diol (25-hydroxycholesterol). This oxysterol is a potent inhibitor of enzymatic activity ($IC_{50} < 1$ μM) (10,12,13, 24). Repression of the synthesis of immunoprecipitable HMG–CoA reductase by this oxysterol has been demonstrated by Faust *et al.* (29) in a Chinese hamster ovary (CHO)-derived compactin-resistant cell (UT-1) line that overproduces the enzyme and by Sinensky *et al.* (30) in CHO cells. Luskey *et al.* (31) have shown that the level of mRNA for the reductase in UT-1 cells is reduced in the presence of the oxysterol. Similar results were obtained by Kandutsch *et al.* (10,12) in wild-type CHO cells (see also Ref. 24). Based on this, Kandutsch *et al.* (32) proposed a model for the regulation of HMG–CoA reductase activity by oxysterols. The oxysterol binds to a cellular protein, and the resultant oxysterol–protein complex then acts to repress transcription of the gene for reductase production. In support of this model, several investigators (23,24,26,33) isolated a protein which exhibits high affinity and low capacity for 25-hydroxycholesterol and other oxysterols which are known to inhibit reductase activity. Although 25-hydroxycholesterol has been isolated from cells, there is no direct evidence that this oxysterol functions as the natural regulator of cholesterol biosynthesis (34). This model (Fig. 1) was in direct contrast to the prevailing view that cholesterol, accumulating in cells after endocytosis of low density lipoprotein (containing cholesteryl esters), is the regulatory messenger molecule (35).

THE DIOXIDOSQUALENE PATHWAY

Side-chain oxysterols are known to be products of primary metabolism in mammalian systems. Derivatives of cholesterol hydroxylated in the 25- or 26-positions are produced in liver during bile acid biosynthesis and side-chain hydroxylation at the 20α- and 22*R*-positions in the initial step of the conversion of cholesterol to steroid hormones in endocrine organs (36–38). Another mode of oxysterol biosynthesis has been described which utilizes the isopentenoid pathway to

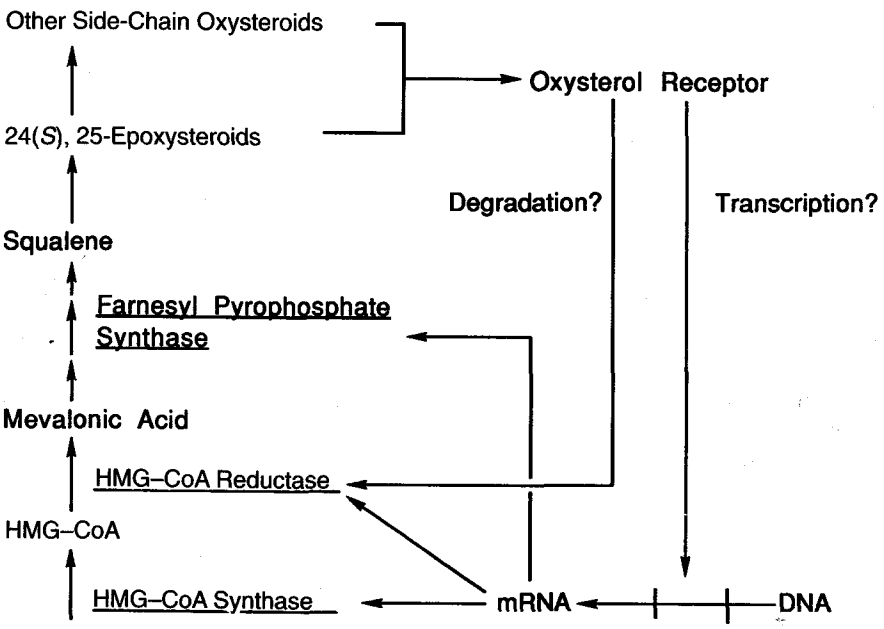


FIG. 1. Kandutsch model for the oxysterol regulation of sterol biosynthesis, adapted from References 44 and 45. HMG–CoA, 3-hydroxy-3-methylglutaryl CoA.

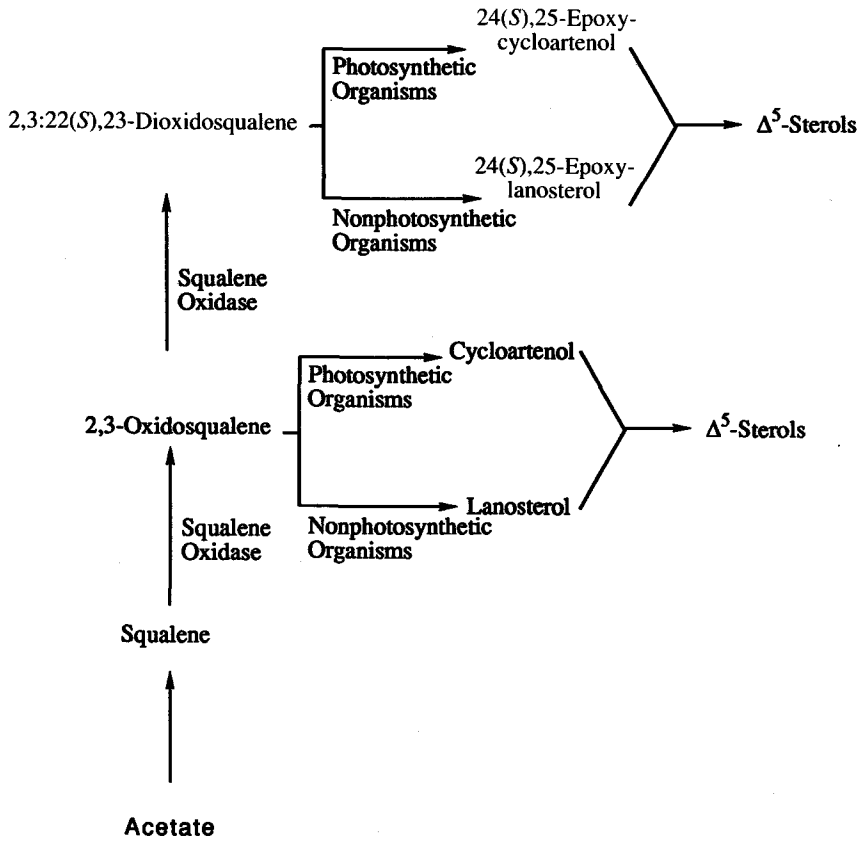


FIG. 2. The biosynthesis of sterols and side-chain oxysterols in plants, animals and microorganisms.

produce side-chain derivatives of cholesterol and lanosterol (Fig. 2) (39,40). Such compounds are derived from squalene 2,3-epoxide by the introduction of a second oxygen function to form 2,3,22,23-dioxidosqualene prior to cyclization. Thus, this intermediate has been shown to form 24(*S*),25-epoxylanosterol, 24(*S*),25-epoxycholesterol, and 25-hydroxycholesterol in mammalian systems (40–43). 24(*S*),25-Epoxycholesterol has been isolated from cultured mouse L cells, Chinese hamster lung fibroblasts, and human liver (34). These oxygenated side-chain derivatives proved to be potent inhibitors of HMG–CoA reductase and sterol biosynthesis, and they possess a high affinity for the oxysterol binding protein (34,40–43). These results add further support to the hypothesis that oxysterols may be natural regulators of cholesterol biosynthesis in mammalian cells (9,12). The dioxidosqualene pathway has recently been reviewed (44,45), as has its occurrence in plants, animals, and microorganisms (46,47), and its evolution (48).

REGULATION OF HMG–CoA REDUCTASE

A key regulatory step in overall sterol biosynthesis is catalyzed by HMG–CoA reductase; therefore, much of the effort toward the development of inhibitors of sterol biosynthesis has focused on this enzyme (3,49,50).

Oxysterols are significant inhibitors of the HMG–CoA reductase found in plant tissues (51–53). However, in intact animal cells, oxysterols are potent inhibitors of reductase activity (8,11,12,23), which requires intact cells and active protein synthesis (54). This effect has recently been attributed to a decrease in the rate of HMG–CoA reductase synthesis (29,30,55) and under some (29,56), but not all (57), conditions to an increase in the rate of enzyme degradation. Side-chain oxysterols, which are products of primary metabolism, are among the most potent inhibitors of enzymatic activity. This high activity would be consistent with their proposed role as natural regulators of cholesterol biosynthesis in mammalian systems.

The inhibitory activity of side-chain oxysterols can vary in different cell lines. In this report we have combined the results from different studies and organized the results according to the type of cell line.

The inhibition of HMG–CoA reductase in L cells (a subline of NCTC clone 929 mouse fibroblasts) by a number of side-chain oxysterols is presented in Table 1, based on published reports (23,34,58). In general, sterols with an intact isooctyl side-chain and a 3β-hydroxyl group showed more potent inhibitory activity (23,34). Also, sterols with a 3-ketone group showed generally somewhat less activity than those with a 3β-hydroxyl group (23). It is known that 3-keto sterols with a sat-

TABLE 1
Side-Chain Oxysterol Inhibition of 3-Hydroxy-3-methylglutaryl CoA Reductase Activity in L Cells[a]

Oxysterols	Inhibition of reductase activity (IC_{50}, μM)
Cholest-5-ene-3β,25-diol	0.17
(25*S*)-Cholest-5-ene-3β,26-diol	0.16
(25*R*)-Cholest-5-ene-3β,26-diol	0.26
Cholest-5-ene-3β,20α-diol	0.30
3β,25-Dihydroxycholest-5-en-7-one	0.48
27-Norcholest-5-ene-3β,20α-diol	0.77
27-Norcholest-5-ene-3β,25-diol	0.86
24(*S*),25-Epoxycholest-5-en-3β-ol	0.89
3β-Hydroxycholest-5-en-24-one	1.0
3β-Hydroxy-27-norcholest-5-en-25-one	1.2
26,27-Bisnorcholest-5-ene-3β,20α-diol	1.2
3β-Hydroxycholest-5-en-22-one	1.4
25-Hydroxycholesta-4,6-dien-3-one	1.8
3β-Hydroxylanost-8-en-24-one	1.8
Cholest-5-ene-3β,22(*S*)-diol	1.9
25-Hydroxycholest-4-en-3-one	3.5
20-Pentylprega-5-ene-3β,20α-diol	5.4
20-Butylprega-5-ene-3β,20α-diol	8.2
25,26,27-Trinorcholest-5-ene-3β,20α-diol	9.7
25-Hydroxycholesta-3,5-dien-7-one	10
20-Propylpregn-5-ene-3β,20α-diol	>30.0
20-Ethylpregn-5-ene-3β,20α-diol	>30.0
20-Methylpregn-5-ene-3β,20α-diol	—
Pregn-5-ene-3b,20α-diol	—

[a]Data from References 23, 34 and 58.

urated A ring are efficiently reduced in L cell cultures to the alcohols while Δ^4-3-ketones are not reduced and accumulate as the conjugated ketones exhibiting less activity (23).

Table 2 presents the results of inhibition studies done on primary cultures of fetal mouse liver cells (34). Although the overall values for reductase inhibition are somewhat lower than those reported for L cells (Table 1), the general trends in inhibitory activity remain similar. Side-chain oxysterols with intact iso-octyl side chains exhibit the greatest inhibition. The most potent oxysterol in these series was 25-hydroxycholesterol.

The effect of side-chain oxysterols on the reductase found in rat intestinal epithelial cells (IEC-6, CRL 1592) are presented in Table 3 (42,59). In these studies, lanost-8-ene-3β,25-diol was the most potent inhibitor of enzymatic activity. Chemical oxidation to the 3-keto derivative resulted in a significant reduction in reductase activity (42). The authors also reported that dioxidosqualene was cyclized to 24(*S*),25-epoxylanost-8-en-3β-ol and metabolized to 24(*S*),25-epoxycholesterol in this cell line (42).

TABLE 2
Side-Chain Oxysterol Repression of 3-Hydroxy-3-methylglutaryl CoA Reductase Activity in Primary Cultures of Liver Cells[a]

Oxysterol	Repression of reductase activity (IC_{50}, μM)
Cholest-5-ene-3β,25-diol	3.0
Cholest-5-ene-3β,20α-diol	3.2
Cholest-5-ene-3β,22(*S*)-diol	5.8
Cholest-5-ene-3β,22(*R*)-diol	7.5
20-Pentylprega-5-ene-3β,20α-diol	10.0
27-Norcholest-5-ene-3β,25-diol	26.0
3β-Hydroxycholest-5-en-24-one	62.0
20-Butylpregn-5-ene-3β,20α-diol	75.0
3β-Hydroxy-27-norchcholest-5-en-25-one	>75.0

[a]Data from Reference 34.

TABLE 3
Side-Chain Oxysterol Repression of 3-Hydroxy-3-methylglutaryl CoA Reductase Activity in Rat Intestinal Epithelial Cells[a]

Oxysterol	Inhibition of reductase activity (IC_{50}, μM)
Lanost-8-ene-3β,25-diol	0.10
24(*S*),25-Epoxylanost-8-en-3β-ol	0.24
24(*S*),25-Epoxycholest-5-en-3β-ol	0.30
25-Hydroxylanost-8-en-3-one	0.30

[a]Data from References 44 and 59.

In other recent studies, (25*R*)-5α-cholest-8(14)-ene-3β,26-diol-15-one was found to be a potent inhibitor of reductase in CHO–K1 cells (60). At a concentration of 0.1 μM, this oxysterol caused a 50% reduction in enzymatic activity. Its potency was indistinguishable from that of its analog lacking the carbon-26 hydroxyl group, 3β-hydroxy-5α-cholest-8(14)-en-15-one. Interestingly, 3β-hydroxy-5α-cholest-8(14)-en-15-one and (25*R*)-cholest-5-ene-3β,26-diol (both potent inhibitors of reductase) have been found to show synergism in the reduction of the levels of reductase activity in CHO-K1 cells (1). When equimolar concentrations of both inhibitors were added together, synergistic reduction of enzyme activity was observed at total oxysterol concentrations of 0.1, 0.2 and 0.5 μM. A maximal synergistic effect in lowering reductase activity (28% greater than that predicted) was observed at 0.1 μM total oxysterol concentration.

CONCLUSIONS

Summarized in this review are studies on the side-chain oxysterol mediated repression of HMG–CoA reductase activity in various mammalian cell lines. These studies have provided much useful information concerning the possible role of side-chain oxysterols as natural regulators of reductase activity and, thus, sterol biosynthesis. The studies have also contributed to our basic understanding of regression of cholesterol biosynthesis in general. Continued basic research in this area should provide further leads for the development of new approaches for the control of cholesterol biosynthesis and the reduction of serum cholesterol levels.

REFERENCES

1. Nes, W.R., and McKean, M.L. (1981) *Biochemistry of Steroids and Other Isopentenoids*, pp. 1–37, University Park Press, Baltimore.
2. Gibbons, G.F., Mitropoulos, K.A., and Myant, N.B. (1982) *Biochemistry of Cholesterol*, pp. 122–142, Elsevier Biomedical, Amsterdam.

3. Goldstein, J.L., and Brown, M.S. (1990) *Nature 243,* 425–430.
4. Lipid Research Clinics Program (1984) *JAMA 251,* 351–364.
5. Lipid Research Clinics Program (1984) *JAMA 251,* 365–371.
6. Blankenhorn, D.H., Nessim, S.A., Johnson, R.L., Sanmarco, M.E., Azen, S.P., and Cashin-Hemphill, L. (1987) *JAMA 257,* 3233–3239.
7. Parish, E.J., Nanduri, U.B.B., Seikel, J.M., Kohl, H.H., and Nusbaum, K.E. (1986) *Steroids 48,* 407–418.
8. Schroepfer, Jr., G.J. (1981) *Ann. Rev. Biochem. 5,* 585–621.
9. Schroepfer, Jr., G.J. (1982) *Ann. Rev. Biochem. 51,* 555–581.
10. Kandutsch, A.A., and Taylor, F.R. (1985) in *Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissues* (Straus, J.F., and Menon, K.M.J., eds.) pp. 194–219, George F. Stickley Co., Philadelphia.
11. Gibbons, G.F. (1983) in *3-Hydroxy-3-methylglutaryl Coenzyme A Reductase* (Sabine, J.R., ed.) pp. 153–232, CRC Press, West Palm Beach.
12. Kandutsch, A.A., Chin, H.W., and Heiniger, H.-J. (1978) *Science 201,* 498–501.
13. Kandutsch, A.A., and Chin, H.W. (1973) *J. Biol. Chem. 248,* 8408–8417.
14. Smith, L.L. (1981) *Cholesterol Autoxidation,* pp. 231–256, Plenum Press, New York.
15. Parish, E.J., Nanduri, U.B.B., Kohl, H.H., and Taylor, F.R. (1980) *Lipids 21,* 27–30.
16. Gibbons, G.F. (1983) *Biochem. Soc. Trans (London) 11,* 649–651.
17. Cheng, K.P. Nagano, N., Bang, L., and Ourisson, G. (1977) *J. Chem. Res. (S)217,* (M)2501–2521.
18. Nagano, J., Poyser, J.P., Cheng, K-P., Bang, L., and Ourisson, G. (1977) *J. Chem. Res. (S)218,* (M)2522–2571.
19. Zander, M., Patrick, K., Bang, L., and Ourisson, G. (1977) *J. Chem. Res. (S)219,* (M)2572–2584.
20. DeFay, R., Astruc, M.E., Roussillon, S., Decomps, B., and Crastes de Paulet, A. (1982) *Biochem. Biophys. Res. Commun. 106,* 362–372.
21. Astruc, M., Laporte, M., Tabacik, C., and Crastes de Paulet, A. (1978) *Biochem. Biophys. Res. Commun. 85,* 691–700.
22. Moriasaki, M., Sonoda, Y., Makino, T., Ohihara, H., Ikekawa, H., and Sato, Y. (1986) *J. Biochem. 99,* 597–600.
23. Taylor, F.R., Saucier, S.E., Shown, E.P., Parish, E.J., and Kandutsch, A.A. (1984) *J. Biol. Chem. 259,* 12382–12387.
24. Gibbons, G.F. (1983) in *3-Hydroxy-3-methylglutaryl Coenzyme A Reductase* (Sabine, L., ed.) pp. 153–233, CRC Press, West Palm Beach.
25. Schroepfer, Jr., G.J., Parish, E.J., Tsuda, M., Raulston, D.L., and Kandutsch, A.A. (1979) *J. Lipid Res. 20,* 994–998.
26. Kandutsch, A.A., Taylor, F.R., and Shown, E.P. (1984) *J. Biol. Chem. 259,* 12388–12397.
27. Sonoda, Y., and Sato, Y. (1983) *Chem. Pharm. Bull. (Japan) 31,* 1698–1701.
28. Sato, Y., Sonoda, Y., Morisaki, M., and Ikekawa, N. (1984) *Chem. Pharm. Bull. (Japan) 32,* 3305–3308.
29. Faust, J.R., Luskey, K.L., Chin, D.J., Goldstein, J.L., and Brown, M.S. (1982) *Proc. Natl. Acad. Sci. USA 79,* 5205–5209.
30. Sinensky, M., Torget, R., and Edwards, P.A. (1981) *J. Biol. Chem. 256,* 11744–11779.
31. Luskey, K.L., Faust, J.R., Chin, D.J., Brown, M.S., and Goldstein, J.L. (1982) *J. Biol. Chem. 258,* 8462–8476.
32. Kandutsch, A.A., Chen, H.W., and Shown, E.P. (1980) *J. Biol. Chem. 255,* 10813.
33. Kandutsch, A.A., Chen, H.W., and Shown, E.P. (1977) *Proc. Natl. Acad. Sci. USA 74,* 2500–2511.
34. Saucier, S.E., Kandutsch, A.A., Taylor, F.R., Spencer, T.A., Phirwa, S., and Gayren, A.K. (1985) *J. Biol. Chem. 260,* 14571–14582.
35. Gould, R.G. (1951) *Am. J. Med. 11,* 209–227.
36. Vlahcevic, Z.R., Schwartz, C.C., Gustaffson, J., Halloran, L.G., Danielson, H., and Swell, L. (1980) *J. Biol. Chem. 255,* 2925–2933.
37. Pederson, J.L., Björkhem, I., and Gustaffson, J. (1979) *J. Biol. Chem. 254,* 6464–6469.
38. Smith, L.O., Teng, J.L., Lin, Y.Y., Seitz, P.K., and McGehee, M.F. (1981) *J. Steroid Biochem. 14,* 889–900.
39. Schroepfer, Jr., G.J., Parish, E.J., Pascal, Jr., R.A. and Kandutsch, A.A. (1980) *J. Lipid Res. 21,* 571–584.
40. Nelson, J.A., Stackbeck, S.R., and Spencer, T.A. (1981) *J. Biol. Chem. 256,* 1067–1068.
41. Panini, S.R., Sexton, R.C., and Rudney, H. (1984) *J. Biol. Chem. 259,* 7767–7774.
42. Paini, S.R., Sexton, R.C., Gupta, A.K, Pansh, E.J., Chitrakorn, S., and Rudney, H. (1986) *J. Lipid Res. 27,* 1290–1294.
43. Spencer, T.A., Gayen, A.K., Phirwa, S., Nelson, J.A., Taylor, F.R., Kandutsch, A.A., and Enckson, S. (1985) *J. Biol. Chem. 260,* 13391–13394.
44. Spencer, T.A. (1994) *Acc. Chem. Res. 27,* 83–90.
45. Taylor, F.R. (1992) in *Regulation of Isopentenoid Metabolism,* ACS Symposium Series 497 (Nes, W.D., and Parish, E.J., eds.) pp. 81–93, American Chemical Society, Washington, D.C.
46. Parish, E.J. (1991) in *Physiology and Biochemistry of Sterols,* (Patterson, G.W., and Nes, W.D., eds.) pp. 324–336, American Oil Chemists' Society, Champaign.
47. Parish, E.J. (1992) in *Regulation of Isopentenoid Metabolism,* ACS Symposium Series 497 (Nes, W.D., and Parish, E.J., eds.) pp. 146–161, American Chemical Society, Washington, D.C.
48. Parish, E.J. (1994) in *Evolution of Natural Products,* ACS Symposium Series 562 (Nes, W.D., ed.) American Chemical Society, Washington, D.C.
49. Brown, M.S., Faust, J.R., Goldstein, J.L., Kaneko, I., and Endo A. (1978) *J. Biol. Chem. 253,* 1121–1132.
50. Nankanishi, M., Goldstein, J.L., and Brown, M.S. (1988) *J. Biol. Chem. 263,* 8929–8938.
51. Bach, T.J., and Lichtenthaler, H.K (1987) in *Ecology and Metabolism of Plant Lipids* (Fuller, G. and Nes, W.D., eds.) ACS Symposium Series 325, pp. 109–139, American Chemical Society, Washington, D.C.
52. Bach, T.J., Wettstein, A., Boronat, A., Ferrer, A., Enjuto, M., Gruissem, W., and Narita, J.O. (1991) in *Physiology and Biochemistry of Sterols* (Patterson, G.W., and Nes, W.D., eds.) pp. 29–49, American Oil Chemists' Society, Champaign.
53. Stermer, B.A., Bianchini, G.M., and Korth, K.L. (1994) *J. Lipid Res. 35,* 1133–1140.
54. Cavenee, W.K., Chen, H.W., and Kandutsch, A.A. (1981) *J. Biol. Chem. 256,* 2675–2681.
55. Chen, H.W., Richards, B.A., and Kandutsch, A.A. (1982) *Biochim. Biophys. Acta 712,* 484–489.
56. Tanaka, R.D., Edwards, P.A., Lan, S.-F., and Fogelman, A.M. (1983) *J. Biol. Chem. 258,* 13331–13339.
57. Sinesky, M., Torget, R., Schnitzer-Polokoff, R., and Edwards, P.A. (1982) *J. Biol. Chem. 257,* 7284–7296.
58. Kandutsch, A.A., and Chen, H.W. (1974) *J. Biol. Chem. 249,* 6057–6061.
59. Panini, S.R., Gupta, A., Sexton, R.C., Parish, E.J., and Rudney, H. (1987) *J. Biol. Chem. 262,* 14435–14440.
60. Schroepfer, Jr., G.J., Kim, H.-S., Vermilion, J.L., Stephens, T.W., Pinkerton, F.D., Needleman, D.H., Wilson, W.K., and St. Pyrek, J. (1988) *Biochem. Biophys. Res. Commun. 151,* 130–136.
61. Pinkerton, F.D., Pelley, R.P., and Shroepfer, Jr., G.J. (1992) *Biochem. Biophys. Res. Commun. 186,* 569–573.

[Received August 22, 1994, and in revised form January 26, 1995; Revision accepted January 26, 1995]

Growth of *Cucurbita maxima* L. Plants in the Presence of the Cycloartenol Synthase Inhibitor U18666A[1]

Gregeory P. Fenner* and Ibrahim Raphiou

Crop Science Department, North Carolina State University, Raleigh, North Carolina 27695

ABSTRACT: Squash, like other Cucurbitaceae, have unique sterol profiles that offer an excellent opportunity to examine the relationship between sterol biosynthesis and plant growth. To determine the effect of sterol biosynthesis inhibition on squash growth, Cucurbita maxima seedlings with and without cotyledons were subjected to increasing concentrations of the cycloartenol synthase (EC 5.4.99.8) inhibitor 3ß-(2-diethylaminoethoxy)androstenone (U18666A). Inhibition of shoot growth was concentration-dependent (from 0, 2, 5, 10, and 20 µM); plants with intact cotyledons grew to 26.4, 23.7, 21.6, 20.0, and 15.6 cm, respectively, at the above inhibitor concentrations, compared to 25.5, 19.4, 17.0, 12.0, and 1.1 cm for plants with severed cotyledons. In plants with severed cotyledons, 10 and 20 µM U18666A caused rapid necrosis of the first two, newly emerged, primary leaves, and halted new leaf formation. Secondary root formation was initially affected at all inhibitor concentrations regardless of whether cotyledons were present or not. Vegetative tissue showed a decrease in the accumulation of the major squash sterol, 7,22-stigmastadienol, accompanied by increased accumulation of minor sterol components. Sterol profiles in cotyledons were unaltered. The data show that sterols are crucial for maintaining plant growth and viability, but do not address the cotyledonary effect on growth with respect to sterol biosynthesis.

Lipids 30, 253–256 (1995).

Inhibitors of plant sterol biosynthesis may be used to determine the importance of sterols in plant growth and development. It is not clear which step in the sterol biosynthetic pathway should be targeted in designing compounds for plant growth regulation. Plant growth can be reduced by hydroxymethylglutaryl CoA (HMG–CoA) reductase (EC 1.1.1.34) inhibitors such as mevinolin (1). However, since HMG–CoA reductase is also involved in the synthesis of other isopentenoids (phytohormones, etc.), further investigations are needed to define the nature of the inhibitory effect. Plants grown in the presence of inhibitors affecting cytochrome-P450-dependent sterol 14α-demethylation show reduced seed germination, reduced plant growth and reduced growth of cells in culture (2,3). Some attention has been given to the design of transition state inhibitors that mimic high-energy intermediates produced during sterol biosynthesis, for example, during C-24 transmethylation and Δ8–Δ7sterol isomerization (4). The inhibition of squalene has been proposed to be a favored target site for inhibition (5). The rationale is that inhibiting this step in sterol biosynthesis should not affect the synthesis of other isopentenoids, as would HMG–CoA reductase inhibitors, or the accumulation of sterol intermediates caused by inhibitors of 14α-demethylation. More thorough reviews on these types of inhibitors and their modes of action have been published (6–8).

As part of our long-range goal of determining the physiological significance of sterols in plant growth, we utilized a well-defined sterol containing plant, zucchini squash *Cucurbita maxima* L., in our experimentation on the effect of the inhibitor 3ß-(2-diethylaminoethoxy)androstenone (U18666A) on whole plant growth. This inhibitor, developed clinically as a hypocholesterolemic agent, has been shown to inhibit squalene cyclization in microsomal preparations from both pea and yeast (5). Bach and Lichtenthaler (9) also showed that it could reduce root growth in radish seedlings. Though sterol transport in plants is questionable, we further asked whether intact cotyledons would have any effect on growth of the plant when new sterol synthesis is inhibited. This forethought is based on the fact that squash have the α/ß duality that is partitioned between vegetative and cotyledonary tissue, respectively (10–13). In this article we report the effect of U18666A on sterol biosynthesis and growth on whole squash plants, and the unexpectedly positive effect of intact cotyledons on sustaining plant growth in the presence of the inhibitor.

MATERIALS AND METHODS

Cucurbita maxima L. (Fordhook zucchini) seeds were purchased from Burpee Seed Company (Warminster, PA). Seeds were sown between germination paper and placed in

[1]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at Crop Science Department, Box 7620, North Carolina State University, Raleigh, NC 27695-7620.

Abbreviations: FID, flame-ionization detection; GC, gas chromatography; HMG–CoA, hydroxymethglutaryl CoA; TMSE, trimethylsilyl ether; U18666A, 3ß-(2-diethylaminoethoxy)androstenone.

Copyright © 1995 by AOCS Press

Hoagland's nutrient solution for four days in a darkened growth chamber. At this time, cotyledons were imbibed, and uniform seedlings were selected. Plants were divided into 25-mL test tubes (three plants per tube × two replicates × two experiments). The test tubes were wrapped in foil, and plants were grown in a plant growth chamber at 23.8°C on a 16/8 h day/night cycle. U18666A was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA) and dissolved in water to produce a 2 mM stock solution. Aliquots were added to Hoagland's to give the following inhibitor concentrations: control (Hoagland's), 2, 5, 10, and 20 μM U18666A. After the first true leaves emerged, cotyledons were removed with a razor from half of the seedlings. From each set of seedlings, three plants per test tube (50 mL) were grown for four weeks using the inhibitor treatments described above.

In a separate study, seedlings with and without cotyledons were grown for two weeks in inhibitor-free Hoagland's medium, and then transferred to 10 μM inhibitor solutions. These plants were then grown for an additional two weeks to test the inhibitor's effect on growth after leaves were established. Finally, to determine the effect of secondary root formation on shoot growth under our experimental conditions, secondary roots from plants grown for one week were severed with a razor, and the primary roots were coated with lanolin to prevent nutrient uptake by the exposed vascular bundles. These plants were grown for an additional three weeks in inhibitor-free Hoagland's medium.

At harvest, plant height, petiole length, and leaf width were measured for each treatment from plants grown with and without cotyledons. Prior to drying and sterol extraction, cotyledons were removed from the set of plants where cotyledons remained intact during the growth period; thus sterols from these plants and their cotyledons were analyzed separately.

Plant material was oven-dried at 80°C for 48 h, weighed, ground with a mortar and pestle, and then extracted with 15 mL of warm chloroform/methanol (2:1, vol/vol) in a water bath. The mixture was filtered through Whatman No. 1 filter paper, and an organic phase was produced by partitioning the filtrate against 2 mL of 1% NaCl, followed by vortexing and spinning at 2,000 rpm. After discarding the aqueous phase, volumes were adjusted with chloroform, an aliquot was removed, and total lipid was determined gravimetrically. Samples were dried under nitrogen and then refluxed with KOH/MeOH/H_2O (20:60:40, wt/vol/vol) containing 0.2% pyrogallol. After cooling, the mixture was extracted three times with diethyl ether, and the ether extracts were pooled and dried under nitrogen. Total sterol fractions were recovered by alumina chromatography using published methods, except that toluene was substituted for benzene (14). Trimethylsilyl ether (TMSE) derivatives of sterols (15) were identified and quantified by gas chromatography (GC) with cholesterol TMSE as an internal standard. The instrument consisted of a Varian 3500 Gas Chromatograph (Sugarland, TX) equipped for flame-ionization detection (FID), and a Varian 8100 autosampler. Data were processed using a Varian Star workstation. Sterols were analyzed on a Supelco (Bellefonte, PA) fused silica SPB-1 capillary column, 30 m × 0.32 mm i.d., 0.25 μ film thickness, under the following conditions: oven temperature, 230°C isothermal; injection port, 280°C; detector, 300°C; split ratio, 18:1; average linear carrier gas velocity (helium), 76.5 cm/s; column head pressure, 33 psi.

RESULTS

Plant growth in the presence of U18666A was inhibited in a concentration-dependent manner (Table 1). Also shown in Table 1 is the effect that cotyledons had on sustaining growth in the presence of the inhibitor. The effect on petiole length and leaf width is most evident. As compared to total plant height, which included root and shoot lengths (much of which was established during germination and prior to introduction of the inhibitor), measurement of petiole length and leaf width showed very well the effect the inhibitor has on leaf formation and growth. The cotyledons had a positive effect on sustaining plant viability. Plants grown in the presence of the inhibitor had more intensely green leaves than did controls.

To test the effect of the inhibitor on growth after leaves had been established, plants with and without cotyledons were grown in inhibitor-free nutrient solution after the initial germination period for up to two weeks. During that growth period, leaves were fully expanded and growth was normal. When plants were subsequently placed in 10 μM inhibitor solution and grown for an additional two weeks, plants with cotyledons grew to 24 cm and those without cotyledons to 22.5 cm, compared to 26.4 and 25.7 cm for controls with and without cotyledons, respectively. In both cases, and especially where cotyledons were severed, leaves began to exhibit necrosis.

Sterol synthesis was altered both qualitatively and quantitatively. Most noticeable was the reduction in the accumulation of 7,22-stigmastadienol and 7-stigmastenol, which are normally the only two predominant sterols in squash vegetative tissue (10). This reduction was accompanied by an in-

TABLE 1
Measurement of Plant Height, Petiole Length, and Leaf Width from Control and Sterol-Inhibited Squash Plants

Treatment (μM inhibitor)	Plant height (cm)	Petiole length (cm)	Leaf width (cm)
With cotyledons			
Control	26.4 ± 0.1	8.7 ± 1.2	5.4 ± 0.8
2	23.7 ± 0.9	7.3 ± 0.9	4.9 ± 0.8
5	21.6 ± 0.7	6.1 ± 0.8	4.4 ± 0.7
10	20.0 ± 0.1	5.6 ± 0.7	4.2 ± 0.7
20	15.6 ± 2.5	3.5 ± 0.5	3.8 ± 0.4
Without cotyledons			
Control	25.7 ± 1.3	8.0 ± 1.5	4.8 ± 0.8
2	19.4 ± 0.4	4.9 ± 0.9	3.5 ± 1.0
5	17.0 ± 1.1	3.4 ± 0,9	3.2 ± 0.6
10	12.0 ± 0.8	n.g.[a]	n.g.
20	11.0 ± 1.0	n.g.	n.g.

[a]n.g., No measurable growth at the end of the growth period.

TABLE 2
Levels[a] of Sterols in Control and Sterol-Inhibited Squash Plants

	Treatment (μM inhibitor)									
Sterol	Control W[b]	2 W	5 W	10 W	20 W	Control W/O[c]	2 W/O	5 W/O	10 W/O	20 W/O
7,22-Stigmastadienol	625	438	272	290	278	803	327	349	662	732
7-Stigmastenol	294	163	142	135	106	335	112	112	112	97
7,25-Stigmastadienol	30	83	95	101	79	47	87	112	82	64
7,24(28)-Stigmastadienol	80	70	41	40	36	105	50	56	26	33
7-Ergostenol	72	178	134	141	151	81	138	163	147	99
Sitosterol	24	71	71	61	73	25	26	54	21	27
Clerosterol	56	89	82	60	81	31	69	74	71	32
Gramisterol	10	68	82	95	69	13	59	69	24	—
Cyclolaudenol	65	50	35	36	28	60	42	43	23	37
Total[d]	137	146	117	115	115	163	114	126	139	122

[a]μg/g Dry weight.
[b]With intact cotyledons.
[c]With severed cotyledons.
[d]Total μg/g dry weight for all sterols (including minor components not shown) ¥ 10.

crease in the levels of other components, especially the 24-methyl sterols, 7-ergostenol, and gramisterol. The changes in individual components and in total sterols that were seen on a dry weight basis are listed in Table 2. Similar results had previously been obtained with celery cells grown in the presence of the 14α-demethylase inhibitor paclobutrazol (2,16). While the synthesis of the two 24α-sterols, 7,22-stigmastadienol and 7-stigmastenol, is apparently decreased, the synthesis of the 24ß-sterol, 7,25-stigmastadienol, seems unaffected. We did not, however, attempt to determine the amount of 7,22,25-stigmastatrienol (24ß) in 7,22-stigmastadienol, which are known to co-elute under these GC conditions. The accumulation of squalene or 2,3-oxidosqualene was not observed in any of the inhibited plants.

We further noted that the changes in sterol composition that coincided with reduced growth were most pronounced in plants in which cotyledons were allowed to remain and growth inhibition was more gradual. In plants without cotyledons at 10 and 20 μM inhibitor concentrations, there was virtually no new tissue growth after germination. However, the data for the latter treatments could be somewhat misleading, especially in regard to the high levels of 7,22-stigmastadienol observed (Table 2), which are likely due to accumulation of this sterol prior to introduction of the seedlings into the inhibitor solution, after which lack of dry matter production resulted in what appears to be increased 7,22-stigmastadienol.

The percentage of each major sterol relative to total sterol found in the cotyledons was qualitatively unchanged (data not shown). The sterol profile is also in agreement with previous data (10), although no efforts were made to determine the amount of the other major cotyledon sterol, 7,22,25-stigmastatrienol.

The initial impact of the inhibitor was on the root system. Regardless of the presence or absence of cotyledons, secondary root growth was inhibited at all inhibitor concentrations. However, when plants had their secondary roots removed and then were grown in inhibitor-free media, we observed that growth for these plants was similar to that of plants whose root systems had remained intact. Thus, it is unlikely that reduced absorption alone was responsible for the diminished growth seen in response to the sterol synthesis inhibitor.

DISCUSSION

It is known that cotyledons function as storage reserves for developing young seedlings, and that early removal of cotyledons from germinated seeds affects growth (17,18). However, the cotyledon effect we observed in regard to sterol synthesis and plant viability is not easily explained. In our experiments on plants on which cotyledons were allowed to remain intact during the growth period, cotyledons were not senesced. The observation that sterol profiles in cotyledons were unaffected by the inhibitor was somewhat expected and may suggest that the sterols in these organs are preformed during seed maturation.

If we assume that intact cotyledons result in an overall greater nourishment for the axis, the presence or absence of cotyledons on control plants should also have resulted in a greater difference in growth of the controls like that seen in inhibited plants (with and without cotyledons), but this was not the case. One explanation for this could be that in inhibited plants on which cotyledons remained intact, the cotyledons may be acting as a sink for the inhibitor, thus reducing its effectiveness. The fact that cotyledons imbibe and expand does not rule out the possibility of inhibitor accumulation in these organs. That inhibition of sterol biosynthesis is involved is further shown by the decreased growth of plants grown for two weeks on inhibitor-free media followed by two weeks on 10 μM inhibitor, regardless of whether the cotyledons were present or not.

Our data do not differentiate between the qualitative and quantitative requirements for sterols to maintain plant growth. The reduction in the amount of total sterol accompanied by

the qualitative changes suggests that in plants, as with yeast, both micro- and macrorequirements are being met by different sterols (19). It is currently well accepted that sterols are required for membrane synthesis and function. Thus the data presented here could be taken to mean that lack of sterol synthesis precludes cell maturity resulting in the inability of the cells to divide or grow. Given that cells in whole plants are under strict hormonal controls relative to both growth and differentiation, it appears that lack of sterol synthesis negates the effect of such signals. Observed lack of secondary root growth even at low inhibitor concentrations suggests that sterols are themselves involved in differentiation, or at least that the lack of sterol and/or membrane synthesis coincides with the inhibition of secondary root formation. Radish seedlings that had been inhibited for sterol synthesis likewise exhibited reduced secondary root formation (9). At these same inhibitor concentrations, however, leaf tissue seems to be able to grow and expand which is likely to be due to an inhibitor gradient in the vascular tissue, while roots are directly exposed. At higher inhibitor concentrations, the damage to the shoots would be expected to be more pronounced, as was observed.

Since no direct sterol transport mechanisms have been proven to exist in plants, an interesting question arises as to the different effects the inhibitor may have on the biosynthesis of 24α- vs. ß-sterols in these plants. This question is raised by data showing that in vegetative tissue the amounts of the 24α-isomers, 7,22-stigmastadienol and 7-stigmastenol, decrease while the amount of 7,25-stigmastadienol is unaffected. Thus for cucurbits, determining the timing of biosynthesis or the organ specific requirements for a given sterol or structural analogue would be significant in determining target sites for sterol inhibition, which may be true for other plant groups as well. It has been shown that the timing of synthesis of certain sterols is correlated to plant developmental events and that different plant parts may contain different kinds and amounts of sterols (10,20–22).

In the current study, it appears that the inhibitor may have had secondary effects in addition to inhibiting cycloartenol synthase (EC 5.4.99.8). The accumulation of 24-methylsterols and 24-methyl-sterol precursors implies that the second C-24 alkylation step might be affected to a lesser degree. Regardless, inhibitors such as U18666A are invaluable since their modifying effects of sterol biosynthesis in plants allows us to gain insights into the roles of these sterols in plant cellular processes. The effects of U18666A we observed on suspension cultures provide us with a direct tool to further study its effect on cell division, growth and membrane composition.

ACKNOWLEDGMENTS

The authors wish to thank Dawn Harrison, USDA Insect Neurobiology and Hormone Laboratory, Beltsville, Maryland, for assistance with the mass spectral identification of sterols. The research was supported in part by the North Carolina Agricultural Research Service.

REFERENCES

1. Bach, T.J. (1986) *Lipids 21*, 82–88.
2. Yates, P.J., Lenton, J.R., and Goad, L.J. (1993) *Pestic. Sci. 39*, 257–265.
3. Khalil, I.A., and Mercer, E.I. (1990) *Phytochemistry 29*, 417–424.
4. Rahier, A., Taton, M., Bouvier-Nave, P., Schmitt, P., Benveniste, P., Schuber, F., Narula, A.S., Cattel, L., Anding, C., and Place, P. (1986) *Lipids 21*, 52–62.
5. Cattel, L., Ceruti, M., Delprino, L., Balliano, G., Duriatti, A., and Bouvier-Nave, P. (1986) *Lipids 21*, 31–38.
6. Burden, R.S., Cooke, D.T., and Carter, G.A. (1989) *Phytochemistry 28*, 1791–1880.
7. Mercer, E.I. (1991) *Lipids 26*, 584–597.
8. Mercer, E.I. (1993) *Prog. Lipid Res. 32*, 357–416.
9. Bach, T.J., and Lichtenthaler, H.K. (1987) *Ecology and Metabolism of Plant Lipids* (Fuller, G., and Nes, W.D., eds.) Vol. 325, pp. 109–139, American Chemical Society, Washington, DC.
10. Fenner, G.P., Patterson, G.W., and Lusby, W.R. (1989) *Lipids 24*, 271–277.
11. Garg, V.K., and Nes, W.R. (1985) *Lipids 20*, 876–883.
12. Sucrow, W., Slopianka, M., and Kircher, H.W. (1970) *Phytochemistry 15*, 1533–1535.
13. Fenner, G.P., and Patterson, G.W. (1992) *Phytochemistry 31*, 73–75.
14. Holden, M.J., and Patterson, G.W. (1982) *Lipids 17*, 215–219.
15. Thompson, Jr., R.C., Patterson, G.W., Thompson, M.J., and Slover, H.T. (1981) *Lipids 16*, 694–699.
16. Haughan, P.A., Burden, R.S., Lenton, J.R., and Goad, L.J. (1989) *Phytochemistry 28*, 781–787.
17. Kutschera, U. (1992) *J. Plant Physiol. 140*, 319–323.
18. Ceccarelli, N., and Lorenzi, R. (1992) *J. Plant Physiol. 140*, 190–194.
19. Rodriguez, R.L., Taylor, F.R., and Parks, L.W. (1982) *Biochem. Biophys. Res. Commun. 106*, 435–448.
20. Fenner, G.P., Patterson, G.W., and Koines, P.M. (1986) *Lipids 21*, 48–51.
21. Heupal, R.C., Sauvaire, Y., Le, Ph., Parish, E.J., and Nes, W.D. (1986) *Lipids 21*, 69–75.
22. Kalinowska, M., Nes, W.R., Crumley, I-.G., and Nes, W.D. (1990) *Phytochemistry 29*, 3427–3434.

[Received June 29, 1994, and in revised form January 21, 1995; Revision accepted January 22, 1995]

Biosynthesis and Distribution of Insect-Molting Hormones in Plants—A Review[1]

John H. Adler[a,*] and Robert J. Grebenok[b]

[a]Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931 and [b]Department of Plant Science, University of Arizona, Tucson, Arizona 85721

ABSTRACT: Insect-molting hormones, phytoecdysteroids, have been reported to occur in over 100 plant families. Plants, unlike insects, are capable of the biosynthesis of ecdysteroids from mevalonic acid, and in several cases the biosynthesis of phytoecdysteroids was also demonstrated to proceed *via* sterols. *Spinacia oleracea* (spinach) biosynthesizes polypodine B and 20-hydroxyecdysone, which is the predominant insect-molting hormone found in plant species. The onset of ecdysteroid production in spinach requires the appropriate ontogenetic development within the plant, which is related to leaf development. In spinach, lathosterol is the biosynthetic precursor to ecdysone and 20-hydroxyecdysone. Phosphorylated ecdysteroid intermediates, particularly ecdysone-3-phosphate, are required during biosynthesis. Polyphosphorylated forms of ecdysteroids are putative regulatory components of the pathway. During spinach development, the 20-hydroxyecdysone is transported from the sites of biosynthesis to the apical regions. An analysis of the physiological data available suggests that different species may synthesize ecdysteroids in various organs and distribute these ecdysteroids to other sites. Annual plants appear to concentrate ecdysteroids in the apical regions, including flowers and seeds. Perennial plants may recycle their ecdysteroids between their deciduous and their perennial organs over the growing season. Further investigations of ecdysteroid biosynthesis and physiology within plants will be required before an acceptable system can be designed to test phytoecdysteroid effectiveness *in vivo* against insect herbivory.

Lipids 30, 257–262 (1995).

The insect-molting hormone, 20-hydroxyecdysone, was first discovered in plants in 1966 and was shown to be structurally identical to the molting hormone isolated from insects (1). Since then over 100 phytoecdysteroid structures have been identified in plants, with 20-hydroxyecdysone being the most common one reported (2,3). The biological activity of some phytoecdysteroids has been tested by a variety of insect bioassays, with many of these structures demonstrating molting hormone activity (4–7). The compounds have been found in various plant structures of both annuals and perennials, including bark and rhizomes (2). Recent studies have demonstrated that active biosynthesis of phytoecdysteroids takes place in young developing tissues (8–11). Transport of ecdysteroids from the site of biosynthesis to other organs has been reported (9,12,13) as well as the apparent cycling of ecdysteroid from deciduous organs in herbaceous perennials (14–17). Ecdysteroids appear to be transported to the growing portions of the plant from their biosynthetic sources. A multiplicity of ecdysteroid conjugates have been observed in plants (2), with recent reports suggesting that phosphorylated conjugates may also be involved in the regulation of ecdysteroid metabolism in spinach (11). The presence of ecdysteroids and their conjugates in plants has been suggested to be a factor which may alter development of nonphytoecdysteroid-adapted insect species (6,7). Hence these compounds may potentially prove useful to control insect herbivory on agriculturally important plants.

PLANT BIOSYNTHETIC STUDIES

The biochemistry of insect-molting hormone has been extensively studied in insects and has been reviewed (18). Insects and all other arthropods require sterols in their diet due to their inability to endogenously cyclize squalene to the requisite sterols needed for membrane function and hormone production, such as insect-molting hormone (19,20). Plants, however, possess a complete sterol biosynthetic pathway, and many are capable of insect-molting hormone biosynthesis. Recent studies on plant ecdysteroid production have shown an active and highly controlled biosynthetic pathway early in plant development as demonstrated by the incorporation of [2-^{14}C]mevalonic acid (MVA) (9–11). The seeds of *Spinacia oleracea*, spinach, and other chenopod plants (Chenopodiaceae) have high levels of ecdysteroid; spinach has 100 μg/g fresh weight and *Chenopodium album* 36–80 μg/g fresh weight (8,21). As the seeds germinate, the level of ecdysteroid decreases on a gram fresh weight basis due to expansion and growth of the plant and the apparent low level of phytoecdysteroid biosynthesis during this stage of development (8). However, in spinach the level of ecdysteroid was never lower than 13 μg/g fresh weight. This is well within the

[1]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at Department of Biological Sciences, Michigan Technological University, 1400 Townsend Dr., Houghton, MI 49931.

Abbreviation: MVA, mevalonic acid.

Copyright © 1995 by AOCS Press

concentration range of phytoecdysteroid dietary intake reported to interfere with the development of susceptible insects (7). An increase in biosynthesis of ecdysteroids occurs in developing spinach plants between days 11 and 20 as demonstrated by [2-^{14}C]MVA incorporation (9) into first leaves. Onset of biosynthesis eventually produces a ratio of ecdysteroid to sterol of 10:1 on a μg/g fresh weight basis as compared to a prior ratio of 1:1 (8,9). In spinach, the flow of sterol into ecdysteroid on a μg/g fresh weight basis is thus a major sink for carbon entering the triterpenoid pathway *via* squalene. Roots are also thought to be a site of biosynthesis in chenopods, since spinach was shown to contain 30 μg/g fresh weight ecdysteroids (Grebenok, R.J., unpublished data), *C. album* fluctuating levels of ecdysteroid (22), and *Kochia scoparia* 14 μg/g of ecdysteroids in these tissues (23). Radiolabel incorporation studies with isolated root systems have not been reported.

Evidence to date suggests that the biosynthesis of ecdysteroids in spinach proceeds as illustrated in Figure 1. The most frequently reported phytoecdysteroid is 20-hydroxyecdysone (2) which possesses a reduced side chain at C-24 (Fig. 1). Many plants have also been reported to possess C-24 alkylated ecdysteroids (for a review, see Ref. 2), and these presumably are biosynthesized from C-24 alkylated sterols. Spinach possesses exclusively Δ^7-sterols which are also found in related chenopods (24) and in some other plant families (25). The C-24 reduced Δ^7-sterol, lathosterol, is a logical precursor available for the biosynthesis of a C-24 reduced side-chain ecdysteroid. Lathosterol conversion to ecdysteroid has clearly been demonstrated by the precursor product relationships reported between the two (10). Particularly compelling evidence for this relationship is seen during ontogeny of the plant (10). When young leaves are newly emerging from the apical meristem, they are incapable of converting [2-^{14}C]MVA to ecdysteroid. In these tissues, the C-24 alkyl sterols are radiolabeled by [2-^{14}C]MVA; however, lathosterol could not be isolated, and ^{14}C label from [2-^{14}C]MVA was not associated with lathosterol, although lathosterol carrier was added (10). Thus, lathosterol biosynthesis in spinach is apparently coordinately regulated with ecdysteroid biosynthesis. Whether such coordinate regulation of C-24 reduction takes place in other plants is yet to be determined. This is important since the majority of plants are known to produce 24-alkylsterols with a C-5(6) double bond, and many of these have been shown to contain small amounts of cholesterol. In species also producing ecdysteroids, the question of whether cholesterol or lathosterol is the preferred substrate for ecdysteroid biosynthesis remains open since both lathosterol and cholesterol could be biosynthesized in such plants. Both cholesterol and lathosterol have been reported to occur in *C. album* and *C. quinoa* (Table 1). Interestingly, [4-^{14}C]cholesterol can be converted to ecdysteroids in spinach when excised leaves are incubated for a prolonged time (10). Whether this is an alternate route or a scavenging pathway, or whether cholesterol can be converted to lathosterol, is yet unknown. The sterol and ecdysteroid compositions of several chenopods are presented in Table 1.

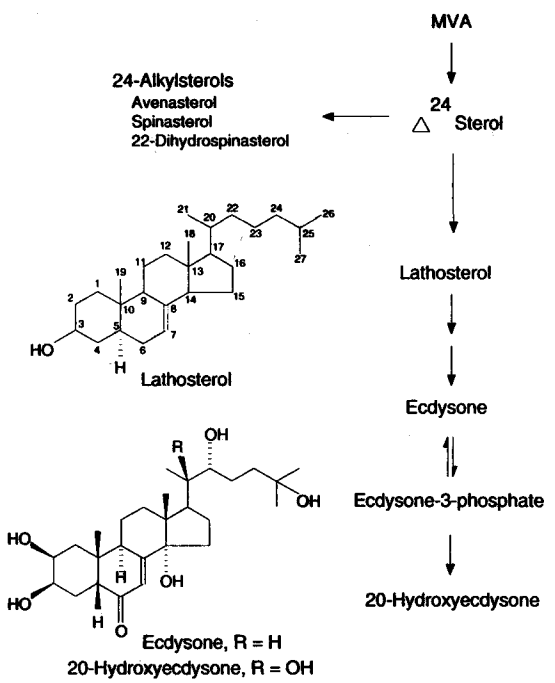


FIG. 1. Biosynthesis of spinach ecdysteroids. The metabolism of mevalonic acid (MVA) proceeds to both 24-alkysterols, lathosterol and subsequently to 20-hydroxyecdysone in intact excised spinach leaves. Ecdysone and ecdysone-3-phosphate are endogenously biosynthesized and metabolized to 20-hydroxyecdysone.

Conversion of a sterol to ecdysteroid requires several structural modifications. Among these are the inversion of the A/B ring juncture, addition of the 6-ketone group, and hydroxylation of the sterol nucleus and side chain. The steps in the bioconversion of sterols to ecdysteroids in plants have been studied only to a limited extent (18). The mechanism of A/B ring juncture inversion in plants is putatively different from that in insects (26). The hydroxylation steps are presumably mediated by cytochrome P-450 monooxygenases as they are in insects (27–29). Recent evidence suggests that phosphate conjugates are important in the processing or targeting of sterols and ecdysteroids in ecdysteroid biosynthesis in spinach. In insects, ecdysteroids are phosphorylated for excretion of the parent ecdysteroid from the insect gut (30,31). In plants, the phosphorylated ecdysteroids appear to be involved in the biosynthetic pathway (11). Spinach endogenously biosynthesizes radiolabeled ecdysone, ecdysone-3-phosphate, and 20-hydroxyecdysone from [2-^{14}C]MVA (11). Incorporation of [22,23-^{3}H]ecdysone and [^{14}C]ecdysone-3-phosphate (biosynthesized from [2-^{14}C]MVA, isolated and purified from spinach) into spinach leaves actively biosynthesizing ecdysteroids produces radiolabeled ecdysone-3-phosphate and 20-hydroxyecdysone from [^{3}H]ecdysone and radiolabeled 20-hydroxyecdysone from [^{14}C]ecdysone-3-phosphate. The production of ecdysone-3-phosphate is a coordinately regulated biosynthetic step in ecdysteroid metabolism in spinach. This is consistent with the lack of production

TABLE 1
Ecdysteroid and Sterol Composition of Some Species in the Chenopodiaceae

Species	Leaf sterols[a]				Ecdysteroid[b] µg/g Fresh weight			
		Percent total sterols by double-bond type			Leaves			
		Δ^7	Δ^5	Δ^0	Lower	Upper	Roots	Reference[c]
Beta vulgaris (Table beet)	24-Alkyl	70	21	—[d]	<0.14[e]		<0.01	24,42
	24-Reduced	—	7[f]	—				*43*
Chenopodium[g] *album*	24-Alkyl	54 (47)	41 (33)	1 (3)	21[e]		ND[h]	24,44,45
	24-Reduced	— (12)[j]	4[f] (5)[f]	— (—)	10[i]	104[i]	8–25[i]	*21–23[i],43*
C. quinoa	24-Alkyl	24	68	—	ND[h]	59[i]	ND[h]	44,46
	24-Reduced	1[i]	7[f]	—				*21[i]*
Kochia scoparia	24-Alkyl	—	100	—	10–11[i]	4[i]	14[i]	24,46
	24-Reduced	—	—	—				*23[i]*
Spinacia oleracea (Spinach)	24-Alkyl	97	—	—	35–69	150	30	10
	24-Reduced	3[i]	—	—				*8*

[a]Reported as percentage of total 4-desmethylsterol. [b]20-Hydroxyecdysone and polypodine B. [c]Reference for ecdysteroids in italics. [d]Not detected is represented by a dash. [e]Analysis of a mixed sample of all leaves. [f]Cholesterol. [g]Data in parentheses from Reference 44. [h]ND, not determined. [i]Data converted to µg/g fresh weight, assuming the plant mass is 90% water. [j]Lathosterol (5α-cholest-7-en-3β-ol).

of ecdysone-3-phosphate in young leaves that are unable to biosynthesize ecdysteroids even when [22,23-^{3}H]ecdysone is provided as a precursor (11). These data, in conjunction with the apparent production of ecdysteroid polyphosphates for the regulation of the ecdysteroid biosynthetic pathway, suggest a new and different role for steroid phosphates in plants compared to insects.

REGULATION OF BIOSYNTHESIS

Onset of ecdysteroid biosynthesis appears to be developmentally controlled with ontogeny, yet a regulatory mechanism for the maintenance of a balanced flow of carbon into ecdysteroids must also exist. Studies designed to investigate the metabolic fate of putative ecdysteroid intermediates in spinach revealed an apparent feedback inhibition of ecdysteroid biosynthesis through the production of ecdysteroid polyphosphates (Fig. 2). When either of the putative intermediates, 2-deoxy-20-hydroxyecdysone or 22,25-dideoxyecdysone, were incubated with spinach leaves actively biosynthesizing ecdysteroids, and this incubation was followed 12 h later by exposure to [2-^{14}C]MVA, a time course of ecdysteroid regulation emerged (11,32). Incubation with either compound caused the disappearance of all 20-hydroxyecdysone mass within the excised leaves and cessation of incorporation of [2-^{14}C]MVA into lathosterol and 20-hydroxyecdysone 24-h post-MVA exposure. Similar incubations with 20-hydroxyecdysone and ecdysone (Fig. 2A) demonstrated depressed incorporation of [2-^{14}C]MVA into 20-hydroxyecdysone, but did not cause the disappearance of 20-hydroxyecdysone mass (11,32). Subsequent analysis revealed that, in the cases where 20-hydroxyecdysone disappeared from the polyalcohol fraction, the 20-hydroxyecdysone had been converted to a polyphosphate. The 20-hydroxyecdysone could be freed from this conjugate by treatment with wheat germ acid phosphatase or human seminal acid phosphatase [EC 3.1.3.2] (11,32). Based on chromatographic evidence, the 20-hydroxyecdysone polyphosphate required a minimal conjugation with four orthophosphates to provide the correct retention values from reverse-phase high-performance liquid chromatography (HPLC). In addition, the 2-deoxy-20-hydroxyecdysone was also polyphosphorylated and was liberated by acid phosphatase treatment from the corresponding incubation with 2-deoxy-20-hydroxyecdysone. At 36-h post-[2-^{14}C]MVA incubation, the endogenous biosynthesis of 20-hydroxyecdysone from [2-^{14}C]MVA appeared to be recovering from the cessation of biosynthesis observed at 24 h. At 36 h, free 20-hydroxyecdysone mass could be isolated from the leaves (11,32), and little 20-hydroxyecdysone polyphosphate was freed by acid phosphatase treatment. The presence of polyphosphorylated ecdysteroids appears to affect the regulation of [2-^{14}C]MVA incorporation into ecdysteroids. High levels of polyphosphorylated ecdysteroids inhibit incorporation, whereas when no polyphosphorylated ecdysteroids can be detected, [2-^{14}C]MVA is incorporated into ecdysteroid end product (Fig. 2). Interestingly, when ecdysone was incubated,

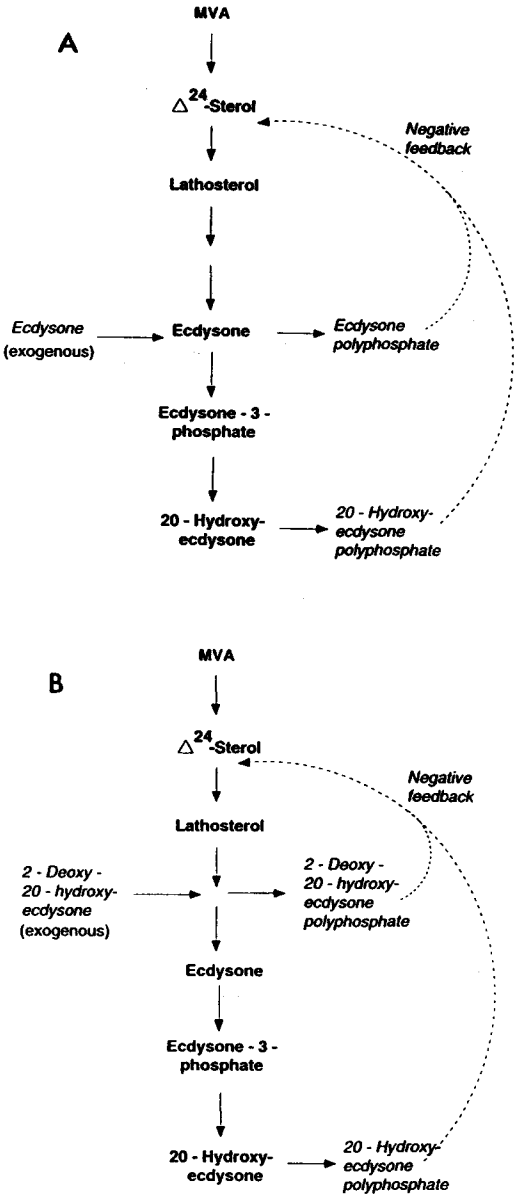


FIG. 2. The metabolism and proposed regulation of the ecdysteroid pathway in spinach. The incorporation of intermediate ecdysteroids—A, ecdysone and B, 2-deoxy-20-hydroxyecdysone—results in the formation of various polyphosphates of the ecdysteroids indicated. The incorporation of MVA into lathosterol and ecdysteroids is also diminished. The polyphosphates are proposed to act through negative feedback to regulate biosynthesis of ecdysteroids. See Figure 1 for abbreviation.

[2-^{14}C]MVA incorporation into 20-hydroxyecdysone was lower, and both free ecdysone and polyphosphorylated ecdysone were observed as well as free 20-hydroxyecdysone (11,32; Fig. 2A). This would suggest that the concentration of ecdysteroid polyphosphate may be sensed as a means to monitor 20-hydroxyecdysone buildup in cells.

The regulation of ecdysteroid biosynthesis *via* ecdysteroid polyphosphates within intact leaves may be essential in controlling ecdysteroid carbon flow within the intact plant. The phytoecdysteroid in spinach appears to be actively transported from the sites of biosynthesis to a sink. Although very young apical spinach leaves show no biosynthesis of ecdysteroid, they show high levels of ecdysteroid (8–10). In intact plants, leaves subtending the very young apical leaves incorporated [2-^{14}C]MVA into ecdysteroids (10). This ^{14}C-labeled ecdysteroid from the subtending leaves appeared in the very young apical leaves over time (9). No [^{14}C]MVA or ^{14}C-labeled sterol was observed in these young apical leaves over the same time course (9). This suggests that ecdysteroid is transported from the sites of biosynthesis, the subtending leaves, to the apical leaves, which act as a sink. Roots may potentially act as a similar sink. Although no data on ecdysteroid biosynthesis in roots is available, spinach roots contain ecdysteroids at levels of about 30 μg/g fresh weight (Grebenok, R.J., unpublished data). Fluctuating levels of ecdysteroids in root tissues have been reported in *C. album* during development (22).

PHYTOECDYSTEROID DISTRIBUTION

The apical-most leaves of spinach contain the highest ecdysteroid levels on a μg/g fresh weight basis (9). During ontogeny of any leaf, the ecdysteroid levels are very high (500–800 μg/g) early in development due to transport, are lower during the stage of active ecdysteroid biosynthesis (100–200 μg/g fresh weight) in that leaf, and are lowest in mature leaves (<100 μg/g fresh weight). Similarly high ecdysteroid levels in the growing tips of plants have been reported in four species of *Chenopodium* (21). Growing tips of *Chenopodium* spp. contain 600–3400 ng ecdysone equivalent per mg dry weight as determined by radioimmunoassay (assuming 90% of plant mass is water, this converts to approximately 60–340 μg/g fresh weight). These chenopods have been reported to possess high levels in the flowers (100–260 μg/g fresh weight), fruits (250–800 μg/g fresh weight), and seeds (250–360 μg/g fresh weight). In the flower organs of *C. album*, the anthers contain four to five times the level of ecdysteroid (500 μg/g fresh weight) reported in the other flower parts. Pollen itself has a low level of ecdysteroid, i.e., <3% of the amount reported for intact anthers. Thus, the anthers, which encase the pollen during development, contain high levels of ecdysteroid whereas the pollen does not. Since individual pollen grains in this wind-pollinated species have a small chance of fertilizing an ovule, these released pollen grains do not need much protection from herbivory. However, since production of pollen grains represents a large investment in energy for the plant, protection of these developing structures by the surrounding anther tissues is an evolutionarily logical defense strategy (21). Interestingly, the three chenopod species which contain little, if any, ecdysteroids in their growing tips (0–3 μg/g fresh weight) produce small amounts of pollen and are presumably insect pollinated (21).

Spinach contains a high level of ecdysteroid (90 μg/g fresh weight) in its fruits, and essentially all (95%) of this ecdysteroid is present in the seed itself (one seed per fruit) with only a small amount present in the fruit walls (8). A related chenopod, *K. scoparia,* also contains 20-hydroxyecdysone

and polypodine B as well as other phytoecdysteroids (23). However, the *Kochia* levels are low compared to those in *C. album* and spinach. *Kochia* contains 20-hydroxyecdysone and polypodine B at the following levels (μg/g fresh weight): leaves, 4–10; roots, 14; seeds, 3; and flower, 5. Interestingly, the distinct concentration gradient seen for ecdysteroids in *C. album* and *S. oleracea* is not observed in the aerial portions of *K. scoparia* (23). *Kochia scoparia* contains the highest levels of ecdysteroids in roots and in the lower and middle leaves of the mature plant. Since *Kochia* is also known to possess other defense compounds including saponins, alkaloids, and phenolic acids (Ref. 23 and refs. therein), ecdysteroids may not be the first line of defense produced by the plant against insects. However, ecdysteroids may serve this function in *C. album* and *S. oleracea*, and thus the distribution within the plant tissues is different.

Ecdysteroids have also been reported to be present in callus and organ tissue cultures of plants known to produce ecdysteroids. A concentration gradient of phytoecdysteroids in the aerial portion of *Ajuga reptans* (13) (Lamiaceae) is similar to the one observed in *S. oleracea* (9) and *C. album* (21). This gradient is most pronounced in wild plants examined in the spring and is less pronounced in plants harvested in summer (33). Plants grown in a greenhouse also maintain a distinct gradient similar to plants collected in the spring in the wild (33). In all cases, the roots of *Ajuga* maintain a high concentration of phytoecdysteroids (1500–3110 μg/g dry weight or 150–311 μg/g fresh weight). The six phytoecdysteroids produced varied in composition depending upon the organ examined and upon growth conditions. Through many subcultures, micropropagated plants of *A. reptans* derived from runners did not vary substantially in total phytoecdysteroids in the aerial parts (33). The roots of micropropagated plants always maintained high levels of phytoecdysteroids (33). When two morphologically different callus culture lines (33) derived from leaves or roots of *A. reptans* were examined, no phytoecdysteroids were found with a lower level of detection of 5 ppm dry weight or 0.5 μg/g fresh weight. The capacity to produce phytoecdysteroids would appear to reside with differentiated cells organized within distinct tissues of *A. reptans*. Phytoecdysteroid production has, however, been reported for some callus cultures of *Achyranthes* spp. (34) (Amaranthaceae), *Trianthema portulacastrum* (35) (Portulacaceae), *Ajuga turkestanica* (36), and hairy root cultures of *A. reptans* var. *atropurpurea* transformed with *Agrobacterium rhizogenes* (37). Analysis of phytoecdysteroid production in *in vitro* root and shoot cultures of *A. reptans* demonstrates that roots are capable of ecdysteroid biosynthesis independent of the shoots (13,38). Similar analyses on shoot cultures devoid of roots demonstrated that shoots did not biosynthesize phytoecdysteroids. The perennial *Serratula tinctoria* (Asteraceae) contains ecdysteroids in leaves, roots, and flowers at 2.6, 8.4, and 12 mg/g dry weight (or 260, 840, and 1200 μg/g fresh weight), respectively. Callus and cell cultures of this plant contain 0.10–0.03 and 0.08 mg/g dry weight (or 100–30 and 8 μg/g fresh weight), respectively, depending upon culture conditions (39). Thus, individual species appear to express their capacity for phytoecdysteroid biosynthesis in different organs, and the state of tissue organization within the plants appears to influence this expression.

Perennial plants appear to accumulate ecdysteroids over many years of growth, whereas annuals bring only the ecdysteroids present in the embryo to the new plant. The high levels of ecdysteroid reported in the fern rhizome *Polypodium vulgare*, 2% dry weight (40) (Polypodiaceae), *Lychnis chalcedonica* root, 0.3% dry weight (17) (Caryophyllaceae), *Serratula tinctoria* root, 1% dry weight (39), and *Rhaponticum carthamoides* (15) (Asteraceae) reflect the accumulation of ecdysteroids in these perennial structures over years. A general pattern of ecdysteroid distribution within plants is emerging, however. The early growth of either newly flushed woody or herbaceous perennials, such as *Taxus* (12) (Taxaceae), *Rhaponticum* (15), *Leuzea* (14) (Asteraceae), *Lychnis* (17), *Serratula* (41), and *Ajuga* (38), contain high levels of ecdysteroids on an ecdysteroid per plant mass basis. With the onset of flowering, *Rhaponticum* and *Serratula* possess high levels in the developing influorescences, and this allows for specific floral parts and seeds and/or fruits to accumulate high levels of phytoecdysteroids. The accumulation in the influorescences is also observed in certain annual plants. *Spinacia* (9) accumulates ecdysteroids in the seed embryo, *C. album* accumulates ecdysteroids in the anthers (21) and intermediate levels are observed to accumulate in flower and seed of *K. scoparia* (23). In annual plants, there appear to be two distinct organs for ecdysteroid biosynthesis, the roots *Kochia* (23) and the leaves *Spinacia* (9); presumably this is also the case for perennial plants. Several plants maintain high levels in the roots *Kochia, Ajuga, Serratula,* and *Rhaponticum* throughout their growth, whereas the levels in the leaves may fluctuate with ontogeny. Trafficking of ecdysteroid within the plant during development is supported by transport of endogenously biosynthesized ecdysteroid observed in *Spinacia* (9) and the shift in ecdysteroid mass profiles observed within perennial plants (12,14,15,17).

CONCLUSION

Ecdysteroids accumulate in various plant organs, including fruits, seeds, flowers, anthers, leaves, and roots, during various phases of their growth. Phytoecdysteroid biosynthesis is dynamic with a specific ontogenetic onset and feedback regulation as demonstrated in *Spinacia* leaves. The herbaceous perennial *Ajuga* biosynthesizes and maintains high levels of ecdysteroids in roots. Other herbaceous perennials accumulate ecdysteroids in root stocks and distribute ecdysteroids to growing aerial structures during ontogeny. These perennials also increase the total ecdysteroid content per plant over many years. However, *Spinacia* and *C. album*, both annuals, produce an increasing concentration gradient of ecdysteroids toward the apex of the plant. Annuals must flower and set seed to maintain their life strategy as species, and seeds carry ecdysteroids forward to developing seedlings. Phytoecdys-

teroids have been suggested to serve as a defense against non-phytoecdysteroid-adapted insects and nematodes (3). Proof of this putative function will require an ability to genetically or physiologically manipulate plant phytoecdysteroid content, to test various hypotheses related to this proposed role.

ACKNOWLEDGMENT

The authors wish to thank the CIBA GEIGY Agricultural Biotechnology Research Unit for generous support of this project.

REFERENCES

1. Nakanishi, K., Koreeda, M., Sasaki, S., Chang, M.L., and Hsu, H.T. (1966) *Chem. Commun.*, 915–917.
2. Horn, D.H.S., and Bergamasco, R. (1985) in *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Kerkut, G.A., and Gilbert, L.I., eds.) Vol. 7, pp. 185–248, Pergamon Press, New York.
3. Lafont, R., and Horn, D.H.S. (1989) in *Ecdysone, from Chemistry to Mode of Action* (Koolman, J., ed.) pp. 39–64, Thieme Medical, New York.
4. Bergamasco, R., and Horn, D.H.S. (1980) in *Progress in Ecdysone Research* (Hoffman, J.A., ed.) pp. 299–324, Elsevier, Amsterdam.
5. Slama, K., Abubakirov, N.K., Gorovits, M.B., Baltaev, U.A., and Saatov, Z. (1993) *Insect Biochem. Molec. Biol. 23,* 181–185.
6. Mele, E., Messeguer, J., Gabarra, R., Tomas, J., Coll, J., and Camps, F. (1992) *Entomol. Exp. Appl. 62,* 163–168.
7. Kubo, I., and Klocke, J.A. (1983) in *Plant Resistance to Insects* (Hedin, P.A., ed.) pp. 329–346, American Chemical Society, Washington, DC.
8. Grebenok, R.J., Ripa, P.V., and Adler, J.H. (1991) *Lipids 26,* 666–668.
9. Grebenok, R.J., and Adler, J.H. (1991) *Phytochemistry 30,* 2905–2910.
10. Grebenok, R.J., and Adler, J.H. (1993) *Phytochemistry 33,* 341–347.
11. Grebenok, R.J., Venkatachari, S., and Adler, J.H. (1994) *Phytochemistry 36,* 1399–1408.
12. Ripa, P.V., Martin, E.A., Cocciolone, S.M., and Adler, J.H. (1990) *Phytochemistry 29,* 425–427.
13. Tomas, J., Camps, F., Coll, J., Mele, E., and Messeguer, J. (1993) *Phytochemistry 32,* 317–324.
14. Varga, E., Szendrei, J., Hajdu, Z., Hornok, L., and Csaki, G. (1986) *Herba Hung. 25,* 115–133.
15. Vereskovskii, V.V., Chekalinskaya, I.I., and Pashina, G.V. (1983) *Rastit. Resur. 19,* 60–65.
16. Yakubova, M.R., and Sakarova, N.A. (1980) *Rastit. Resur. 16,* 98–100.
17. Zibareva, L.N., and Sviridova, T.P. (1989) *Rastit. Resur. 25,* 561.
18. Rees, H.H. (1985) in *Comprehensive Insect Physiology Biochemistry and Pharmacology* (Kerkut, G.A., and Gilbert, L.A., eds.) Vol. 7, pp. 249–293, Pergamon Press, New York.
19. Nes, W.R., and McKean, M.L. (1977) *Biochemistry of Steroids and Other Isopentenoids*, pp. 411–533, University Park Press, Baltimore.
20. Nes, W.R., and Nes, W.D. (1980) *Lipids in Evolution*, pp. 97–155, Plenum Press, New York.
21. Dinan, L. (1992) *Experientia 48,* 305–308.
22. Dinan, L., Riseborough, S., Brading, M., Clement, C.Y., Witts, D.J., Smith, J., Colombe, S., Pettitt, V., Wheeler, D.A., and Greenwood, D.R. (1991) in *Insect Chemical Ecology* (Hrdy, I., ed.) pp. 215–220, Academia Prague and SPB Academic Publishers, The Hague.
23. Dinan, L. (1994) *J. Chromatogr. 658,* 69–76.
24. Salt, T.A., and Adler, J.H. (1985) *Lipids 20,* 594–601.
25. Nes, W.R. (1977) *Adv. Lipid Res. 15,* 233–324.
26. Rees, H.H., Davies, T.G., Dinan, L.N., Lockley, W.J.S., and Goodwin, T.W. (1980) in *Progress in Ecdysone Research* (Hoffman, J.A., ed.) pp. 125–139, Elsevier, Amsterdam.
27. Mitchell, M.J., and Smith, S.L. (1986) *Insect Biochemistry 16,* 525–538.
28. Smith, S.L., and Mitchell, M.J. (1986) *Insect Biochemistry 16,* 49–55.
29. Weirich, G. (1985) *Methods Enzymol. 111,* 454–458.
30. Isaac, R.E., and Slinger, A.J. (1989) in *Ecdysone, from Chemistry to Mode of Action* (Koolman, J., ed.) pp. 250–251, Thieme Medical Publishers, New York.
31. Hoffman, J.A., and Lagueux, M. (1985) in *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Kerkut, G.A., and Gilbert, L.I., eds.) Vol. 1, pp. 435–460, Pergamon Press, New York.
32. Grebenok, R.J. (1993) Studies of Ecdysteroid Biosynthesis in Spinach (*Spinacia oleracea* L.), Ph.D. Thesis, Michigan Technological University, Houghton, pp. 1–136.
33. Camps, F. and Coll, J. (1993) *Phytochemistry 32,* 1361–1370.
34. Hikino, H., Jin, H., and Takemoto, T. (1971) *Chem. Pharm. Bull. 19,* 438–439.
35. Ravishankar, G.A., and Metha, A.R. (1971) *J. Nat. Prod. 42,* 152–158.
36. Lev., S.V., Zakirova, R.P., Saatov, Z., Gorovits, B., and Abubakirov, N.K. (1990) *Khim. Prir. Soedin. 1,* 51–52.
37. Matsumoto, T., and Tanaka, N. (1991) *Agric. Biol. Chem. 55,* 1019–1025.
38. Tomas, J., Camps, F., Claveria, E., Coll, J., Mele, E., and Messeguer, J. (1992) *Phytochemistry 31,* 1585–1591.
39. Corio-Costet, M.F., Chapuis, L., Mouilett, J.F., and Delbecques, J.P. (1993) *Insect Biochem. Molec. Biol. 23,* 175–180.
40. Jizba, J., Herout, V., and Sorm, F. (1967) *Tetrahedron Lett. 18,* 1689–1691.
41. Kholodova, Y.D., Baltaev, U., Volovenko, V.O., Gorovits, M.B., and Abubakirov, N.K. (1979) *Khim. Prir. Soedin 2,* 171–174.
42. Adler, J.H., and Salt, T.A. (1983) *Lipids 18,* 229–232.
43. Ripa, P.V. (1987) Phytoecdysteroid Identification and Quantitation from *Taxus cuspidata, Beta vulgaris, Spinacea oleracea* and *Chenopodium album*, M.S. Thesis, Michigan Technological University, Houghton, pp. 1–77.
44. Xu, S., Patterson, G.W., Lusby, W.R., Schmid, K.M., and Salt, T.A. (1990) *Lipids 25,* 61–64.
45. Adler, J.H., and Salt, T.A. (1987) in *Metabolism, Structure and Function of Plant Lipids*, (Stumpf, P.K., Mudd, J.B., and Nes, W.D., eds.) pp. 119–121, Plenum Publishing Corp., New York.
46. Salt, T.A., Xu, S., Patterson, G.W., and Adler, J.H. (1991) *Lipids 26,* 604–613.

[Received June 15, 1994, and in revised form January 9, 1995; Revision accepted January 17, 1995]

Sterol Metabolism in the Tobacco Hornworm, *Manduca sexta*—A Review[1]

James A. Svoboda* and Gunter F. Weirich

Insect Neurobiology & Hormone Laboratory, ARS, USDA, Beltsville, Maryland 20705

ABSTRACT: A number of intermediates involved in the dealkylation and conversion of the major C_{28} and C_{29} phytosterols to cholesterol in insects were first isolated and identified in studies with the tobacco hornworm, *Manduca sexta,* carried out in our laboratory. We also investigated the effects of a variety of known sterol metabolism inhibitors in *Manduca,* particularly those affecting the Δ^{24}-sterol reductase enzyme, and synthesized and tested a number of new inhibitors as well. In-depth studies of ecdysteroids in *Manduca* during embryogenesis and during pupal–adult development provided new information on molting hormone content, biosynthesis, and metabolism. In addition, this insect has been utilized in the study of three specific enzyme systems of ecdysteroid metabolism, namely 20-monooxygenase, 3-epimerase, and phosphotransferase, which are critical to activation and deactivation of molting hormones in insects.

Lipids 30, 263–267 (1995).

The tobacco hornworm, *Manduca sexta*, has been one of the primary experimental insects utilized in our studies on the elucidation of the pathways of sterol metabolism in insects and is widely used in many laboratories throughout the world for basic studies in virtually every area of insect physiology and biochemistry. This species is particularly useful because a satisfactory artificial agar-based diet is available for rearing and can be modified for metabolic studies. *Manduca sexta* grows quite rapidly, is relatively large, and yields mature larvae which weigh 10 g each about 15 d after egg hatching.

Interest in research on sterol utilization and metabolism in insects was stimulated by the discovery that insects, unlike vertebrates and plants, are unable to biosynthesize the tetracyclic steroid skeleton (1). Sterols are essential as structural components of membranes of insect cells and tissues, as precursors of the ecdysteroids (the only functional steroid hormones yet known in insects), and perhaps as components of other, as yet unknown physiological systems (2). Thus, insects require an exogenous or dietary source of sterol to support normal growth, development, and reproduction. With only a few known exceptions, like the dietary requirement of a Δ^7-sterol in *Drosophila pachea* (3) and a beetle, *Xyleborus ferrugineus* (4), cholesterol will satisfy the dietary need for sterols in insects. However, phytophagous insects often encounter little or no cholesterol in their food plants, and most species have adapted by converting C_{28} and C_{29} phytosterols to cholesterol *via* dealkylation of these phytosterols at C-24 (5). Most phytophagous insects can produce sufficient cholesterol in this manner to satisfy their needs. There are a number of interesting variations in the dealkylation process in certain species, and some phytophagous species totally lack the capability to dealkylate (6).

In addition to sections on the utilization and metabolism of neutral sterols with special emphasis on dealkylation mechanisms, this review will include an outline of ecdysteroid (molting hormone) metabolism in *Manduca*. However, this is not intended as a comprehensive coverage of the topic as we will concentrate on ecdysteroid research done with *Manduca* in our laboratory. For a more thorough coverage of ecdysteroid biosynthesis and metabolism, the reader is referred to several recent reviews by Rees (7), Thompson *et al.* (8), and by Grieneisen (9).

Some of the unique features of sterol metabolism in insects offer potential targets for insect control. Disruption of the conversion of phytosterols to cholesterol, the conversion of cholesterol to the ecdysteroids, or other processes in ecdysteroid metabolism and function would inhibit normal insect development in the early larval instars, which is highly desirable for management of many pest species. Therefore, studies on the effect of inhibitors of steroid metabolism done in *Manduca* will also be included in this review.

DEALKYLATION OF C_{28} AND C_{29} PHYTOSTEROLS

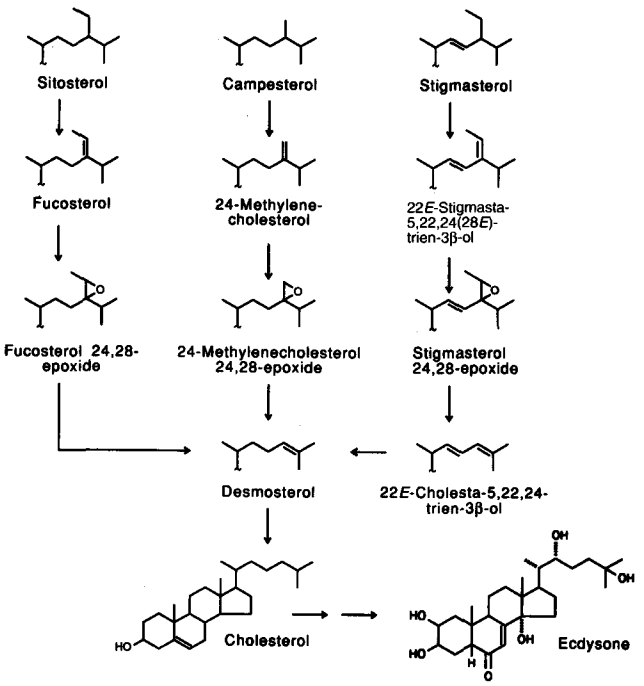
The dealkylation of ergosterol to form 22-dehydrocholesterol in the German cockroach, *Blattella germanica*, was the first conclusive proof of dealkylation of a phytosterol in an insect (10). Later studies with the same insect provided the first unequivocal proof of the dealkylation and conversion of a C_{29} phytosterol to cholesterol in an insect (11). In metabolic studies with the tobacco hornworm, a variety of dietary C_{28} and C_{29} phytosterols were shown to be converted efficiently to cholesterol. Highly purified sitosterol, campesterol, stigmas-

[1]Based on a paper presented at the symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.

*To whom correspondence should be addressed at the Insect Neurobiology & Hormone Laboratory, USDA, ARS, PSI, Bldg. 467, BARC-East, Beltsville, MD 20705-2350.

Abbreviation: 26E26P, 26-hydroxyecdysone-26-phosphate.

Copyright © 1995 by AOCS Press



SCHEME 1

terol, brassicasterol, 22,23-dihydrobrassicasterol, 24-methylenecholesterol, and fucosterol were dealkylated when fed to hornworms, and cholesterol was the major metabolite in each case (12,13). Thus, ethyl, methyl, methylene, and ethylidene groups could all be dealkylated from C-24, and, when present, the Δ^{22}-bond was reduced (Scheme 1).

The first identification of a dealkylation intermediate was accomplished in studies with *Manduca*. Desmosterol ($\Delta^{5,24}$-cholestadienol) was isolated and identified as the terminal intermediate in the conversion of [^{3}H]sitosterol to [^{3}H]cholesterol (12). Desmosterol was subsequently shown to be a common intermediate in the conversion of a variety of C_{28} and C_{29} phytosterols to cholesterol in *Manduca* (13). The Δ^{24}-sterol reductase, the enzyme catalyzing the conversion of desmosterol to cholesterol, was demonstrated in *Manduca* midgut tissue *in vitro* with microsomal preparations, which efficiently converted [^{14}C]desmosterol to cholesterol (14). The midgut microsomal preparation provided the first *in vitro* system for the study of specific sterol conversions in insects and proved useful for studying both normal sterol metabolism and the effects of inhibitors on the 24-reductase enzyme.

The conversion of sitosterol to cholesterol in *Manduca* proceeds *via* fucosterol (24-ethylidenecholesterol) as the initial intermediate (15). In an analogous pathway, [^{3}H]campesterol is converted to [^{3}H]cholesterol with [^{3}H]24-methylenecholesterol as the expected intermediate (16). Similarly, 24-methylenecholesterol is an intermediate in the conversion of dihydrobrassicasterol, the 24*S*-methyl isomer of campesterol, to cholesterol in this insect (16), although 24*S*-methyl isomers are not as efficiently converted to cholesterol as are 24*R*-alkyl sterols.

In contrast to the results with *Manduca*, the omnivorous German cockroach is unable to reduce the Δ^{22}-bond of ergosterol and, presumably, other Δ^{22}-sterols (10). A new sterol, 22*E*-cholesta-5,22,24-trien-3ß-ol, was identified as another intermediate in the conversion of [^{3}H]stigmasterol to cholesterol in the hornworm and in several other species (17). In addition, we established that the Δ^{22}-bond is reduced before the Δ^{24}-bond and that the hornworm can reduce the Δ^{22}-bond only if the Δ^{24}-bond is present. The conversion of 22*E*-cholesta-5,22,24-trien-3ß-ol to desmosterol and cholesterol was also accomplished *in vitro* with the microsomal preparation from hornworm midgut, which, apparently in addition to Δ^{24}-reductase, also has Δ^{22}-reductase activity (17). Stigmasta-5,22*E*,24(28*E*)-trien-3ß-ol was isolated from another lepidopteran, *Spodoptera littoralis*, as a metabolite of stigmasterol, indicating that the stigmasterol dealkylation pathway starts with a conversion analogous to the sitosterol–fucosterol conversion (18). The involvement of epoxides as intermediates following fucosterol and 24-methylenecholesterol in the dealkylation pathway was first demonstrated in the silkworm, *Bombyx mori* (19,20). Studies with *S. littoralis* also showed an epoxide, stigmasterol-24,28-epoxide, as an intermediate in stigmasterol dealkylation (18). The fucosterol 24,28-epoxide lyase enzyme from *Manduca* midgut was shown to have a great preference for 24*R*,28*R* over 24*S*,28*S*-diastereomeric forms of the epoxides (21).

INHIBITORS OF STEROL METABOLISM IN *MANDUCA SEXTA*

The discovery that desmosterol was the terminal intermediate in the dealkylation and conversion of phytosterol to cholesterol prompted us to test the effects of certain hypocholesterolemic agents developed by pharmaceutical firms. These drugs were known to inhibit the Δ^{24}-sterol reductase in vertebrates, where desmosterol is the terminal intermediate in the *de novo* biosynthesis of cholesterol. We first tested triparanol and a number of azasteroids, including 22,25-diazacholesterol (Fig. 1), and found them to inhibit both the Δ^{24}-reductase and larval development in *Manduca* and in other species when fed in a diet containing dietary phytosterols such as sitosterol (22). These inhibitors were also useful for the identification of desmosterol as a common intermediate in the metabolism of C_{28} and C_{29} phytosterols to cholesterol (13), and for the isolation of other intermediates of phytosterol dealkylation in the hornworm and other insects and nematodes (23). Some azasteroids also disrupt other aspects of steroid metabolism, as indicated by the observation that in certain species cholesterol does not reverse the effects of the inhibitor. Through structure–activity relationship studies, it was possible to design and synthesize simpler, more active azasteroids such as 25-azacholestane and 25-azacoprostane, which were over 200 times as active as the azasterols of our original work (24). In some species, including *Manduca*, 25-azacholestane and 25-azacoprostane were inhibitory at dietary concentrations of 1 ppm or less.

Because inhibitors with a complete steroid nucleus would be expensive to synthesize and could present regulatory prob-

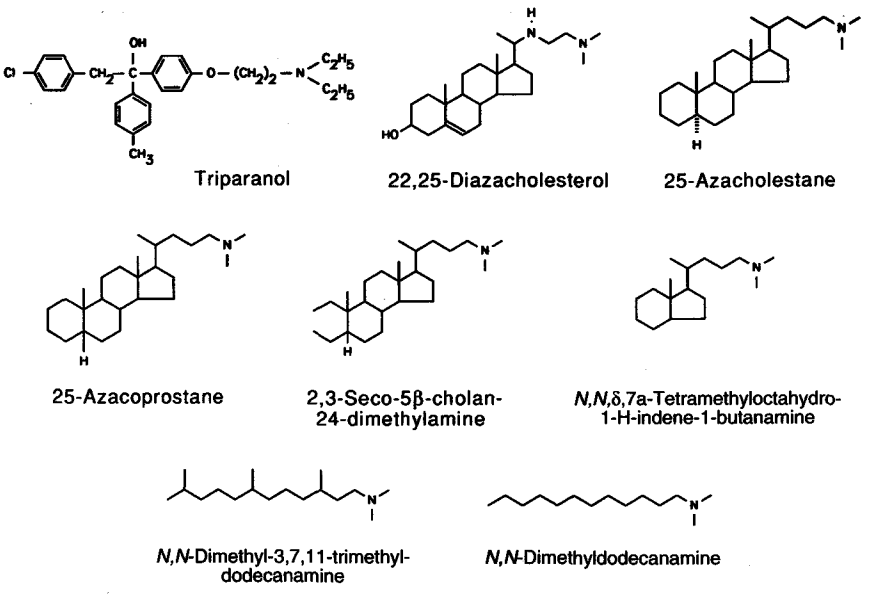


FIG. 1. Representative Δ^{24}-sterol reductase inhibitors.

lems if used as control agents, we attempted to develop non-steroidal compounds that might have the same types of activity as the azasteroids. By opening the A ring, we found that the intact steroid nucleus was not necessary and that even a simple bicyclic compound with the C and D rings and a 25-aza side chain had effects similar to the intact azasteroids. Indeed, simple branched- and straight-chain amines, such as *N,N*-dimethyldodecanamine also inhibited the Δ^{24}-reductase and larval development in *Manduca* and in other species, but at a lower level of activity than the best of the azasteroids (25). Interestingly, all of these Δ^{24}-reductase inhibitors have a dimethylamine functional group. These latter branched- and straight-chain amines were very inexpensive and available in large quantities, and had low vertebrate toxicity. Although these compounds were not suitable for use against phytophagous insects because of their phytotoxicity, they have shown promise for use against other arthropods and certain nematodes (23). Also, these inhibitors have served as valuable tools in our sterol metabolism and endocrinological studies, particularly in our search for new intermediates of dealkylation, as certain intermediates accumulate when later steps in the metabolic pathway are blocked (23).

ECDYSTEROIDS IN *MANDUCA SEXTA*

Although cholesterol has been known to be a precursor of ecdysone since 1963 (26), even before the structure of ecdysone was known, the pathway from cholesterol to ecdysone is still not entirely clear (9). 7-Dehydrocholesterol was first implicated as the primary intermediate between cholesterol and ecdysteroids in *Calliphora stygia* (27). The prothoracic glands are the major site of ecdysteroid biosynthesis in larval insects, and in *Manduca* these glands were found to contain relatively high levels of 7-dehydrocholesterol (9).

Manduca sexta was one of the first insect species from which both ecdysone and 20-hydroxyecdysone were isolated and identified (Fig. 2) (28). 20-Hydroxyecdysone is the most active molting hormone in most larval insects. Both ecdysteroids occur at fairly high titers in seven-day-old *Manduca* pupae. Much smaller quantities of a more polar ecdysteroid, later shown to be 20,26-dihydroxyecdysone, are also present but had a much lower level of biological activity (29). 20,26-Dihydroxyecdysone becomes much more prominent five days later in pupal–adult development, exceeding the levels of ecdysone and 20-hydroxyecdysone. The observed increase in the proportion of 20,26-dihydroxyecdysone lends support to the conclusion that 26-hydroxylation is a mechanism of deactivation. Lesser amounts of 3-epiecdysone, 3-epi-20-hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone were also present in this later developmental stage, and they too are believed to be inactivation products (30). 3-Epiecdysone and 3-epi-20-hydroxyecdysone were initially isolated from hornworm meconium, the metabolic waste products discharged at adult emergence (31). In a complementary study, an enzyme system from *Manduca* midgut was discovered that converted ecdysone to 3-epiecdysone (32).

26-Hydroxyecdysone was first isolated and identified as the major ecdysteroid in tobacco hornworm eggs (33). *Manduca sexta* eggs appear to be unique in this respect. [^{14}C]Cholesterol was injected into late pupae to study ecdysteroid metabolism during the 96-h embryonic development of *Manduca* (34). Samples of eggs were collected at six time intervals after oviposition and the ^{14}C-labeled ecdysteroids were examined in detail. The total ecdysteroid content was quite constant throughout embryonic development (~40 μg/g), and considerable metabolism occurred during this period even though there was little or no *de novo* biosynthesis. In newly laid eggs, 26-hydroxyecdysone-26-phosphate (26E26P) was

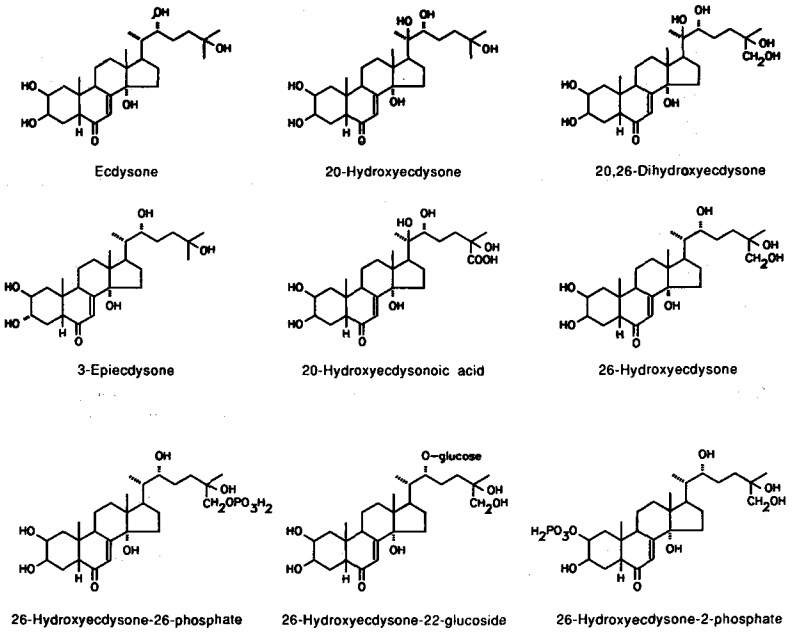


FIG. 2. Representative ecdysteroids.

the major ecdysteroid. Little ecdysone or 20-hydroxyecdysone was detected throughout embryogenesis. The 26E26P was hydrolyzed, and the 26-hydroxyecdysone was metabolized along several pathways, including glucosylation, epimerization, and oxidation to acids, all of which are presumed to be inactivation processes. Conjugates (phosphates and glucosides) constituted the major ecdysteroids throughout embryonic development. The most abundant ecdysteroid isolated from newly emerged first instar larvae was 26-hydroxyecdysone 22-glucoside. The results of these metabolic studies with [^{14}C]cholesterol closely paralleled the results of another study on ecdysteroid metabolism in *Manduca* embryos, in which identification and quantification were based on radioimmunoassay with two different antisera specific for different regions of the ecdysteroid molecule (35).

In another study, newly molted fifth-instar male *Manduca* larvae were injected with [^{14}C]cholesterol and analyzed for their ^{14}C-labeled ecdysteroids at five time intervals between 0 and 16 d after pupation (36). *De novo* biosynthesis of ecdysteroids occurred during the first four days following pupation. The total ecdysteroid levels increased from about 2.5 to 6.5 μg/g, which is much lower than the titers found in *Manduca* eggs. Ecdysteroid metabolism throughout pupal–adult development was characterized by hydroxylation, oxidation, epimerization, and phosphorylation. Concentrations of specific ecdysteroids varied throughout pupal–adult development, but the major metabolites were 3-epi-20-hydroxyecdysonoic acid and phosphoconjugates of 3-epi-20-hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone. Thus, inactivation of ecdysteroids during pupal–adult development in *Manduca* is accomplished by oxidation and epimerization leading to 3-epiecdysteroid acid, as well as by phosphorylation leading to 3-epiecdysteroid phosphates. Interestingly, a considerable amount of ecdysteroids was synthesized during the first four days of pupal–adult development, whereas little or no *de novo* biosynthesis occurred during embryogenesis.

Our laboratory has used *M. sexta* for extensive *in vitro* studies of three specific enzyme systems involved in ecdysteroid metabolism: 20-monooxygenase (EC 1.14.99.22), 3-epimerase, and phosphotransferase (37). Two different ecdysone 20-monooxygenases have been discovered in the midgut, one located in the mitochondria, the other in the microsomes (38,39). These enzymes convert ecdysone to 20-hydroxyecdysone and therefore represent a molting hormone activating enzyme system. Ecdysone 20-monooxygenases are cytochrome P450-dependent enzymes, require oxygen and NADPH as a hydrogen donor, and are inhibited by carbon monoxide. High activity has been found in late fifth-instar larvae and early prepupae, coinciding with or slightly preceding the major increase in hemolymph 20-hydroxyecdysone titer. The interaction between the mitochondrial and the microsomal 20-monooxygenase systems and their contribution to the ecdysteroid balance of the hornworm are not fully understood.

Inactivation of ecdysteroids through 3-epimerization of ecdysone occurs *via* a two-step reaction, an oxidation to 3-dehydroecdysteroids followed by a stereospecific reduction to 3α-hydroxyecdysteroids. Enzymes for these conversions are located in the midgut cytosol (40,41). The first step is catalyzed by ecdysone oxidase (EC 1.1.3.16), the second step by 3-oxoecdysteroid 3α-reductase. The midgut cytosol also contains another enzyme, 3-oxoecdysteroid 3β-reductase, that converts the intermediate (3-dehydroecdysteroid) back to the 3β-hydroxyecdysteroid. Thus, the total amount of ecdysteroid converted to the 3-epimer depends on the balance between the two competing enzymatic reactions.

As mentioned before, another means of inactivation of ecdysteroids involves formation of phosphoconjugates. These have been found in larval, pupal, and pharate adult stages of

development in various species (8). Phosphotransferases in midgut cytosol preparations from *Manduca* have been shown to convert ecdysone to four different phosphoconjugates, and 3-epiecdysone to two phosphoconjugates (42).

The intent of this brief review is to summarize primarily our laboratory's research related to sterol metabolism in *Manduca.* Much of the information presently available on steroid metabolism in insects was initially obtained in studies with this species. Certainly, much has been accomplished using other species as well, but *Manduca* continues to be a major experimental animal for many studies on insect physiology and biochemistry, including those related to new approaches to insect control. For example, much of the initial work on ecdysone agonists, such as RH-5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine), was done with the hornworm (43). Currently, a related benzoylhydrazine (RH-5992) that is very effective against Lepidoptera is being developed commercially (44). Related research on insect sterol metabolism with other species has been described in several recent reviews (e.g., Refs. 6,7,9,45). Continued basic research in these areas of insect steroid biochemistry may provide further leads for the development of new control technology. For example, isolation of specific enzymes involved in steroid metabolism and cloning of the corresponding genes could provide invaluable new tools for researchers in this area. This has been and continues to be an exciting and fruitful area of research in insect biochemistry.

REFERENCES

1. Bloch, K., Langdon, R.G., Clark, A.J., and Fraenkel, G. (1956) *Biochim. Biophys. Acta 21*, 176.
2. Robbins, W.E., Kaplanis, J.N., Svoboda, J.A., and Thompson, M.J. (1971) *Ann. Rev. Ent. 16*, 53–72.
3. Heed, W.B., and Kircher, H.W. (1965) *Science 149*, 758–761.
4. Chu, H.M., Norris, D.M., and Kok, L.T. (1970) *J. Insect Physiol. 16*, 1379–1387.
5. Svoboda, J.A., and Thompson, M.J. (1985) in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* (Kerkut, G.A., and Gilbert, L.I., eds.) Vol. 10, pp. 137–175, Pergamon, New York.
6. Svoboda, J.A., and Feldlaufer, M.F. (1991) *Lipids 26*, 614–618.
7. Rees, H.H. (1989) in *Ecdysone* (Koolman, J., ed.) pp. 152–160, Georg Thieme Verlag, New York.
8. Thompson, M.J., Weirich, G.F., and Svoboda, J.A. (1990) in *Morphogenetic Hormones of Arthropods: Discoveries, Syntheses, Metabolism, Evolution, Modes of Action, and Techniques* (Gupta, A.P., ed.) pp. 325–360, Rutgers University Press, New Brunswick.
9. Grieneisen, M.L. (1994) *Insect Biochem. Molec. Biol. 24*, 115–132.
10. Clark, A.J., and Bloch, K. (1959) *J. Biol Chem. 234*, 2589–2593.
11. Robbins, W.E., Dutky, R.C., Monroe, R.E., and Kaplanis, J.N. (1962) *Ann. Ent. Soc. Am. 55*, 102–104.
12. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1967) *Life Sci. 6*, 395–404.
13. Svoboda, J.A., and Robbins, W.E. (1968) *Experienta 24*, 1131–1139.
14. Svoboda, J.A., Womack, M., Thompson, M.J., and Robbins, W.E. (1969) *Comp. Biochem. Physiol. 30*, 541–549.
15. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1971) *Nature (New Biol.) 230*, 57–58.
16. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1972) *Lipids 7*, 156–158.
17. Svoboda, J.A., Hutchins, R.F.N., Thompson, M.J., and Robbins, W.E. (1969) *Steroids 14*, 469–476.
18. Svoboda, J.A., Rees, H.H., Thompson, M.J., and Hoggard, N. (1989) *Steroids 53*, 329–343.
19. Ikekawa, N., Morisaki, M., Ohtaka, H., and Chiyoda, Y. (1971) *Chem. Commun.*, 1498.
20. Ikekawa, N., Fujimoto, Y., Takasu, A., and Morisaki, M. (1980) *Chem. Commun.* 709–711.
21. Prestwich, G.D., Angelastro, M., DePalma, A., and Perino, M.A. (1985) *Anal. Biochem. 151*, 315–326.
22. Svoboda, J.A., and Robbins, W.E. (1967) *Science 156*, 1637–1638.
23. Svoboda, J.A., and Chitwood, D.J. (1992) in *Regulation of Isopentenoid Metabolism* (Nes, W.D., Parish, E.J., and Trzaskos, J.M., eds.) ACS Symposium Series No. 497, pp. 205–218, American Chemical Society, Washington, D.C.
24. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1972) *Lipids 7*, 553–556.
25. Robbins, W.E., Thompson, M.J., Svoboda, J.A., Shortino, T.J., Cohen, C.F., Dutky, S.R., and Duncan, O.J. III (1975) *Lipids 10*, 353–359.
26. Karlson, P., and Hoffmeister, H. (1963) *Hoppe-Seylers Z. Physiol. Chem. 331*, 298–300.
27. Galbraith, M.N., Horn, D.H.S., and Middleton, E.J. (1970) *Chem. Commun.* 179–180.
28. Kaplanis, J.N., Thompson, M.J., Yamamoto, R.T., Robbins, W.E., and Louloudes, S.J. (1966) *Steroids 8*, 605–623.
29. Thompson, M.J., Kaplanis, J.N., Robbins, W.E., and Yamamoto, R.T. (1967) *Chem. Commun.*, 650–653.
30. Kaplanis, J.N., Thompson, M.J., Dutky, S.R., and Robbins, W.E. (1979) *Steroids 34*, 333–345.
31. Thompson, M.J., Kaplanis, J.N., Robbins, W.E., Dutky, S.R., and Nigg, H.N. (1974) *Steroids 24*, 359–366.
32. Nigg, H.N., Svoboda, J.A., Thompson, M.J., Kaplanis, J.N., Dutky, S.R., and Robbins, W.E. (1974) *Lipids 9*, 971–974.
33. Kaplanis, J.N., Robbins, W.E., Thompson, M.J., and Dutky, S.R. (1973) *Science 180*, 307–308.
34. Thompson, M.J., Svoboda, J.A., Lozano, R., and Wilzer, K.R. (1988) *Arch. Insect Biochem. Physiol. 7*, 157–172.
35. Warren, J.T., Steiner, B., Dorn, A., Pak, M., and Gilbert, L.I. (1986) *J. Liquid Chromatogr. 9*, 1759–1782.
36. Lozano, R., Thompson, M.J., Svoboda, J.A., and Lusby, W.R. (1989) *Arch. Insect Biochem. Physiol. 12*, 63–74.
37. Weirich, G.F. (1989) in *Ecdysone* (Koolman, J., ed.) pp. 174–180, Georg Thieme Verlag, New York.
38. Mayer, R.T., Svoboda, J.A., and Weirich, G.F. (1978) *Hoppe-Seylers Z. Physiol. Chem. 359*, 1247–1257.
39. Weirich, G.F., Svoboda, J.A., and Thompson, M.J. (1985) *Arch. Insect Biochem. Physiol. 2*, 385–396.
40. Weirich, G.F., and Svoboda, J.A. (1992) *Arch. Insect Biochem. Physiol. 21*, 91–102.
41. Weirich, G.F., Feldlaufer, M.F., and Svoboda, J.A. (1993) *Arch. Insect Biochem. Physiol. 23*, 199–211.
42. Weirich, G.F., Thompson, M.J., and Svoboda, J.A. (1986) *Arch. Insect Biochem. Physiol. 3*, 109–126.
43. Wing, K.D., Slawecki, R.A., and Carlson, G.R. (1988) *Science 241*, 470–472.
44. Heller, J.J., Mattioda, H., Klein, E., and Sagenmuller, A. (1992) *Brighton Crop Protection Conference—Pests and Diseases*, The British Crop Protection Council, pp. 59–65.
45. Svoboda, J.A. (1994) *Biochem. Soc. Trans. 22*, 635–641.

[Received July 23, 1994, and in revised form December 19, 1994; Revision accepted December 27, 1994]

Quantitation of Steryl Ferulate and *p*-Coumarate Esters from Corn and Rice[1]

Robert A. Norton*
USDA, ARS, NCAUR,
Mycotoxin Research, Peoria, Illinois 61604

ABSTRACT: The principal steryl ferulate and *p*-coumarate esters of different fractions from processed corn brans and corn oils, unrefined and refined, and from rice bran and rice bran oil were quantified by high-performance liquid chromatography. The results show that hexane-extracted corn oils yield more than five times the amount of esters compared to expeller processed oils. The yields of esters from bran and related products ranged from 0.07 to 0.54 mg/g of bran. Unrefined corn oils had levels from 0.18 to 8.6 mg/g for oil from hexane-extracted bran. By comparison, rice bran had ester levels of 3.4 mg/g of bran, and rice bran oil had levels of 15.7 mg/g of oil. The predominant esters from corn were sitostanyl and campestanyl ferulate, and sitostanyl and campestanyl *p*-coumarate. The principal esters from rice bran were cycloartenyl, 24-methylenecycloartanyl, and campesteryl ferulate. Rice bran oils had low levels of 24-methylenecycloartanyl but high levels of cyclobranol esters. The data presented provide a direct comparison of steryl ferulate and *p*-coumarate levels in the two cereals, and will aid in selecting the most suitable sources for the isolation of these compounds from corn products.
Lipids 30, 269–274 (1995).

Sterols esterified to cinnamic acid and its derivatives (CAD) are found in cereals and other plant families (1,2). The ferulates of cyclic dimethyl and desmethyl sterols occur in rice (3) and are collectively termed γ-oryzanol. Ferulate and *p*-coumarate esters of desmethyl sterols are found in corn, rye, wheat, and triticale (1). Two aspects of steryl CAD esters in corn and related cereals are of interest, namely the possible involvement of these compounds in the susceptibility, or resistance, of preharvest corn to pathogens and whether these compounds might have effects similar to those of rice bran oil or γ-oryzanol on certain types of hypercholesterolemia.

No specific function for these compounds in the plant has been demonstrated, however, one possibility is that they may affect the response of the seed to pathogens, either positively or negatively. The compounds are localized in corn kernels in the inner pericarp and aleurone layer (1), and ferulic acid, the principal compound esterified to the sterols, has been shown to inhibit growth and aflatoxin production in *Aspergillus flavus* (4,5), which is the primary source of aflatoxin contamination in preharvest corn (6). It was anticipated that hydrolysis of the ester by infecting fungi would release inhibitory levels of ferulic acid within the region of the kernel in which they occur. Recent evidence indicates that steryl CAD esters increase the amount of aflatoxin produced by *A. flavus* grown on a synthetic medium but did inhibit spore germination of *Scerlotinia sclerotiorum*, which is not a pathogen of corn but is a pathogen in other plants (R. Norton, and P. Dowd, unpublished data).

Rice bran oil has been reported in a number of studies to be effective in lowering blood cholesterol in some types of hypercholesterolemia, and γ-oryzanol has been suggested as the active factor (7). Similar studies have not been reported on this ester fraction from cereals other than rice. The levels of steryl CAD esters from intact whole corn were low compared with those from rice bran oil, i.e., about 46 μg/g for corn kernels (1) vs. 3500 μg/g for rice bran (8). Localization of the esters in the pericarp and aleurone layers indicated that bran could be a more efficient source of these compounds than whole corn and a recent report showed that one corn bran product contained about twice the level of the steryl CAD as did whole corn, and it contained at least 16 identifiable steryl esters (9). Even a doubling of the concentration of esters by using bran would still give a low yield for corn compared to rice. Because the pericarp and aleurone layers comprise most of the bran fraction, but make up only about 5% of the kernel (10), a yield of 20 times the kernel level would seem to be the upper limit, i.e., about 900 μg/g of bran. To determine which corn products might be the best source for these compounds, it would be helpful to obtain enough of the 16 or more minor constituents of bran for testing against corn pathogens as well as for testing their efficacy in the treatment of hypercholesterolemia.

In the studies reported here, we analyzed different bran fractions for composition and yield of steryl CAD esters and compared these to data obtained on unrefined and refined corn oils. The composition and levels of esters in rice bran and rice bran oil have been reported by others (7,8,11), but samples of

[1]Based on a paper presented at the Symposium on the "Regulation of Biosynthesis and Function of Isopentenoids," Atlanta, Georgia, May 1994.
*Address correspondence at USDA, ARS, NCAUR, 1815 N. University, Peoria, IL 61604.

Abbreviations: CAD, cinnamic acid derivative; CV, coefficient of variation; HPLC, high-performance liquid chromatography.

Copyright © 1995 by AOCS Press

these products were also included in the present study to permit direct comparisons of data obtained by the same analytical methods. Nothing is known to date about the biosynthesis of these compounds. It would also be of interest to know whether esters from different parts of the corn kernel have similar ester levels and compositions. The specific ester compositions of the fractions would be important for evaluating the physiological effects of these compounds from corn in comparison to those from rice.

MATERIALS AND METHODS

Corn bran fractions designated raw bran, bran duster thrus, bran after duster, finished bran, fine high-fiber corn bran (No. 97800), standard corn bran (No. 96530), and ultra-high fiber corn bran (No. 99030) were obtained from Illinois Cereal Mills (Paris, IL). Corn oil processing intermediates designated expeller cake, crude corn oil (expeller extracted), and crude corn oil (hexane-extracted) were obtained from Lauhoff Grain Co. (Danville, IL). Commercial oils and rice bran from the following manufacturers were obtained from local health food stores: Spectrum Naturals, Inc. (Petaluma, CA); Select Origins Inc. (Southampton, NY); California Naturals (The California Olive Oil Corp., Salem, MA); BioSan (New Dimensions Distributors, Fountain Hills, AZ); NOW Foods (Glendale Heights, IL); Hain Pure Food Corp. (Los Angeles, CA); Mazola corn oil (CPC International Inc., Englewood Cliffs, NJ). γ-Oryzanol (as γ-Oryzanol), was purchased from CTC Organics (Atlanta, GA).

Extractions. Aliquots of bran and expeller cake were weighed and dried at 100°C to constant weight to determine percent moisture. Duplicate samples equivalent to 50 g of dry weight were mixed with 250 mL hexane, allowed to set, and extracted twice overnight in the dark. The samples were filtered through a Büchner funnel with filter paper, and the cake was rinsed twice with 125 mL of hexane. The combined extracts were evaporated under reduced pressure at 40°C and stored at −20°C along with the commercial oils prior to use.

Sample preparation. Free fatty acids were removed from samples by adding 3 mL of 1,2-dichloroethane (Sigma, St. Louis, MO) to a 1-g sample of the oil obtained by hexane extraction of bran or the oil obtained from the manufacturer. Next, 1.5 mL of a saturated aqueous solution of Na_2CO_3 (40%, wt/vol) was added to the diluted sample and mixed thoroughly. After centrifuging the mixture on a laboratory centrifuge for several minutes, the organic layer was pipetted off. The aqueous phase was extracted with an additional 3 mL of 1,2-dichloroethane, and the combined organic phases were evaporated to dryness with a stream of N_2 at ambient temperature (12). The dry extract was redissolved in 1 mL of hexane/Et_2O (9:1, vol/vol). A silica gel column was prepared by adding 10 g of silica gel (63–200 μm mesh; Alltech Associates, Deerfield, IL) made up in hexane/Et_2O (9:1, vol/vol) to a 50-mL glass column (28 × 100 mm; Kontes Glass, Flex-Column, Vineland, NJ). The sample was pipetted onto the column and eluted with Solvent Systems S_1, i.e., 50 mL hexane/Et_2O (9:1, vol/vol); S_2, i.e., 30 mL hexane/Et_2O (7:3, vol/vol); and then S_3, i.e., 50 mL hexane/Et_2O (5:5, vol/vol). The final 10 mL of S_2 and the entire volume of S_3 were collected as 10-mL fractions. The fractions were evaporated under reduced pressure and brought up to 1 mL with 2-propanol. The presence or absence of ferulates and *p*-coumarates in the column eluates was monitored by measuring absorption at 327 nm, using a Beckman DU 60 spectrophotometer (Beckman Instruments, Fullerton, CA). Triglycerides, sterol fatty acid esters, and fatty acids were eluted in S_1 and in the first 20 mL of S_2. Steryl CAD was eluted in the last 10 mL of S_2 and S_3. Sterols were not removed, but these were eluted ahead of the steryl CAD esters and did not absorb at the wavelength used for monitoring esters. Examination of all fractions eluted from the silica gel column showed that no appreciable amounts of esters were lost due to premature elution or due to incomplete elution.

High-performance liquid chromatography (HPLC). The steryl CAD mixture obtained was analyzed by HPLC using a C18 reversed-phase (5 μm, 300 Å, Deltabond ODS; Keystone Scientific, Bellefonte, PA) column (4.6 × 250 mm) and acetonitrile/*n*-butanol/acetic acid/water (82:3:2:13, by vol) as solvent system at a flow rate of 1.2 mL/min at ambient temperature. The effluent was monitored using a diode-array detector at 325 nm. Corn components were identified spectroscopically and by their retention times relative to sitostanyl ferulate, and were quantified as previously reported (9). Rice and γ-oryzanol compounds were identified by mass spectrometry using authentic sterols standards as described (9). For each type of sample, each HPLC peak that was included in the calculations was further examined to verify that the respective spectra reasonably matched those of the major ferulate and *p*-coumarates. For corn samples, these were sitostanyl ferulate and sitostanyl *p*-coumarate; for rice, it was cycloartenyl ferulate. Some of the refined corn oils had retention times close to those of *p*-coumarate and ferulate of the crude oils, but the spectra were atypical, and thus those peaks were not included in the calculations. The yield of oil per unit of extracted source material is unknown for the commercial or process oils; therefore yields for bran samples are not given. Each bran sample was extracted twice, and two silica gel column preparations made for one of the extracts to assess within-sample reproducibility for the cleanup procedure. Quantitation and composition data are the averages of duplicate extraction analyses and of the single sample analysis. Two of each of the commercial oil samples (identified by source in the tables) were prepared and analyzed, and the averages are given. The average coefficient of variation (CV) within samples for the composition of the major components was 1.4% for sitostanyl ferulate, 2.4% for campestanyl ferulate, and 12.2% for sitosteryl *p*-coumarate. The average CV for the composition between samples was 1.9, 5.2, and 13.7%, respectively. The average CV for the quantitation within samples (oil basis) was 5.8, 5.5% between samples, and 3.0% for the amount of oil extracted.

RESULTS AND DISCUSSION

Analysis. Use of the Deltabond column gave better resolution of the esters in less time than the column used in previous work (9). The flexibility of the column is illustrated in Figure 1, showing that γ-oryzanol chromatographed with increasing amounts of water in the mobile phase. Similar results were obtained with the ester fraction from corn. Peak numbers 2, 3, 4, and 9 (17.5%) showed increasing resolution as the amount of water was increased; however, peak number 6 (12.5%) was gradually lost under these conditions. For samples of this ester composition, 12.5–15% water seemed optimal as all peaks can be seen at least as shoulders. Water at 13% gave the best resolution for corn esters; a typical chromatogram is shown in Figure 2. Three other C_{18} columns all gave results similar to those in Figure 1 using the base solvent system and run times from 30–70 min. However, there was better separation of peak numbers 8 and 9. Peak numbers 2, 3, and 4 appeared to be ferulates with an unidentified steryl portion. The Deltabond column offers considerable flexibility because a higher percentage of water can be used without substantially lengthening run times.

Composition and level of esters. The results of the analyses for corn materials are given in Table 1, and for rice in Table 2. Representative structures are shown in Scheme 1. Sample R, ConAgra corn bran flour, had been kept for over two years in a −20°C freezer; thus some deterioration may have occurred over time. A previous examination of this sample had given a value of 93.3 μg steryl CAD esters per gram of bran (9). The unrefined corn oils I and W were from the same manufacturer but had different labels on the bottles, similarly for samples U and V.

As is shown in Table 1, significant differences in the yields of esters from different brans and bran processing fractions were seen both on the basis of the amounts of esters per unit of bran as well as for the related yield for oil. The composi-

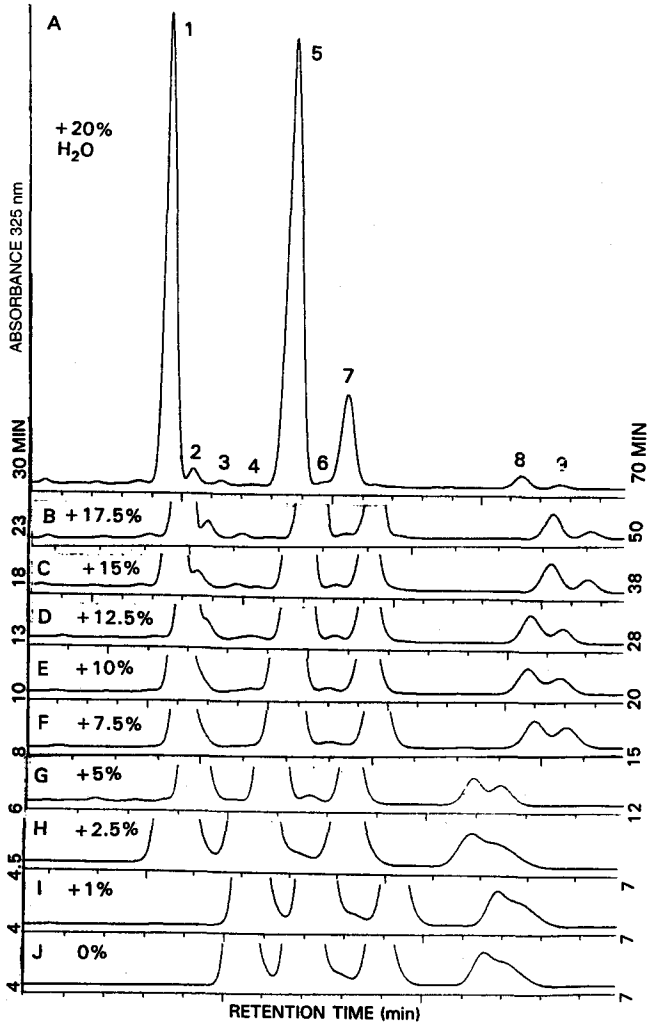


FIG. 1. Effect of increasing water concentration on the separation of γ-oryzanol components on a Deltabond C18 high-performance liquid chromatography column (Keystone Scientific, Bellefonte, PA) using acetonitrile/*n*-butanol/acetic acid/water (94:3:2:1, by vol) with additional water as shown. Detector wavelength, 325 nm. Peak 1, cycloartenyl ferulate; peaks 2, 3, 4, and 9, ferulates, sterol identity unknown; peak 5, 24-methylenecycloartanyl ferulate; peak 6, cyclobranyl ferulate; peak 7, campesteryl ferulate; peak 8, sitostanyl ferulate; not shown (off scale), cycloartanyl ferulate.

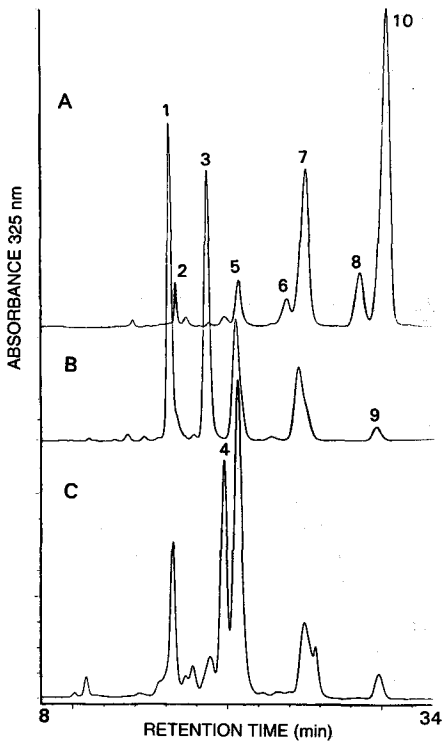


FIG. 2. Representative high-performance liquid chromatography chromatograms of the steryl ferulate and *p*-coumarate fractions of the corn and rice materials investigated. A. Ultra-high fiber bran. B. Rice bran extracted with hexane by author. C. Typical profile for commercial rice bran oils. Peak 1, cycloartenyl ferulate; peak 2, ferulate, sterol identity unknown; peak 3, 24-methylenecycloartanyl ferulate; peak 4, cyclobranyl ferulate; peak 5, campesteryl ferulate; peak 6, campestanyl *p*-coumarate; peak 7, campestanyl ferulate; peak 8, sitostanyl *p*-coumarate; peak 9, cycloartanyl ferulate; peak 10, sitostanyl ferulate.

TABLE 1
Steryl Ferulate and *p*-Coumarate Esters in Corn and Corn Oils[a]

Code	Source	Oil[b] content (g)	Total ester (mg ester/g) [c]Bran	Total ester (mg ester/g) Oil	Composition (wt%) Δ^0-Sito fer	Δ^0-Campe fer	Δ^0-Sito *p*-cou	Δ^0-Campe *p*-cou	Other fer	Other *p*-cou
Corn bran and related fractions										
A	Expeller cake	2.51[d]	0.09	1.8	51.2	25.2	7.6	3.9	10.6	1.5
B	Raw bran	1.78[d]	0.22	6.3	63.5	27.9	1.1	0.4	6.8	0.3
C	Bran after duster	0.77[d]	0.24	15.8	65.5	24.4	1.8	0.7	7.0	0.6
D	Bran duster thrus	2.31[d]	0.54	11.5	62.1	27.2	1.4	0.5	8.3	0.5
E	Finished bran	0.93[d]	0.26	13.2	64.1	26.0	1.0	0.9	7.5	0.5
F	Ultra-high fiber bran	0.49[d]	0.23	23.9	69.4	21.6	1.6	0.6	6.5	0.3
G	Standard corn bran	0.77[d]	0.41	26.3	83.3	11.4	2.0	0.8	2.0	0.5
H	Fine high-fiber bran	0.75[d]	0.36	24.1	83.4	8.4	2.0	0.7	5.5	trace
R	Corn bran flour, ConAgra	0.88[d]	0.07	3.9	85.4	7.4	3.4	1.3	2.4	0.4
Corn oils, unrefined										
I	Spectrum Naturals	[e]		1.2	58.3	24.1	4.5	1.7	10.4	1.0
W	Spectrum Naturals	[e]		1.1	66.7	20.4	2.9	1.8	7.1	1.1
J	Lauhoff Grain Co.	[f]		8.6	59.3	27.3	2.0	0.9	9.9	0.6
K	Lauhoff Grain Co.	[e]		1.5	63.1	25.3	2.9	1.6	6.2	0.9
U	COOC, 32 oz	[e]		0.22	63.4	15.1	6.2	3.2	12.1	trace
V	California Naturals (COOC), 16 oz	[e]		0.18	64.0	22.9	2.3	0.8	9.1	0.9
Corn oils, refined										
S	Mazola	[g]	0.02	100	[h]—	—	—	—	—	
T	Hain Pure Food Co.	[e]	0.02	71.0	29.0	—	—	—	—	

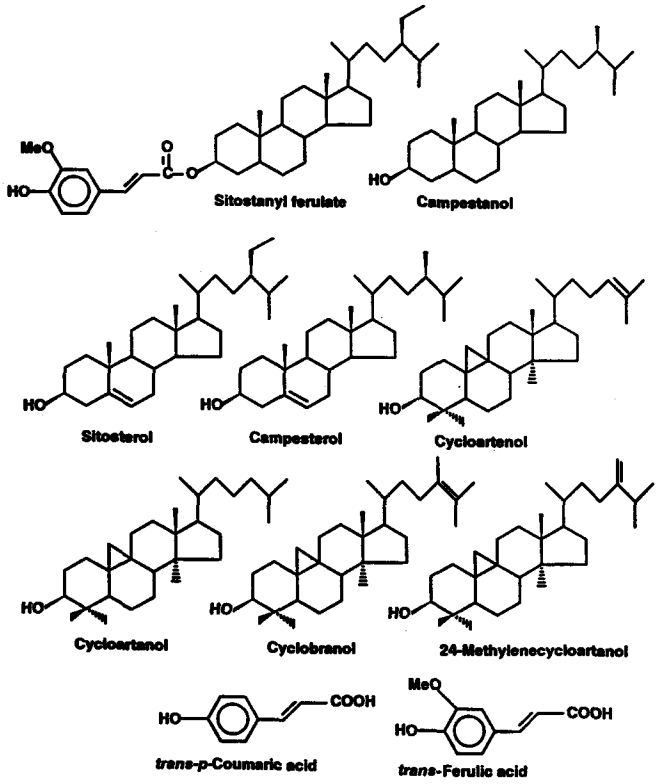
[a]Δ^0-Sito, sitostanyl; Δ^0-Campe, campestanyl; fer, ferulate; *p*-cou, *p*-coumarate; trace (<1%); COOC, The California Olive Oil Corp. (Salem, MA); ConAgra (ConAgra Grain Processing, Omaha, NE); Product A: Lauhoff Grain Co. (Danville, IL); Products B–H: Illinois Cereal Mills (Paris, IL); Spectrum Naturals (Petaluma, CA); Lauhoff Grain Co.; Mazola (CPC International, Inc., Englewood Cliffs, NJ); Hain Pure Food Corp. (Los Angeles, CA).
[b]Per 50 g (dry weight) bran.
[c]Dry weight basis.
[d]Hexane extracted by author.
[e]Expeller extracted by manufacturer.
[f]Hexane extracted by manufacturer.
[g]Extraction method unknown.
[h]Not quantified, interfering compounds.

TABLE 2
Steryl Ferulate Esters in Rice Bran and Rice Bran Oils[a]

Code	Source	Oil[b] content (g)	Total ester (mg ester/g) [c]Bran	Total ester (mg ester/g) Oil	Composition (wt%) Cycloartenyl fer	24-methyleneCA fer	Cyclobranol fer	Sito fer	Campe fer	Other fer
N	Rice bran, NOW Foods	10.66[d]	3.4	15.7	29.4	28.9	trace	14.1	21.9	5.7
L	Rice bran oil, Select Origins	[e]		0.4	13.4	5.2	21.5	9.7	35.2	15.0
M	Rice bran oil, BioSan	[f]		3.0	27.4	4.8	20.1	10.6	24.2	12.9

[a]24-MethyleneCA, 24-methylenecycloartanyl; Sito, sitosteryl; Campe, campesteryl; fer, ferulate; trace, less than 2% of total; NOW Foods (Glendale Heights, IL); Select Origins (Southampton, NY); BioSan (New Dimensions Distributors, Fountain Hills, AZ).
[b]Per 50 g (dry weight) bran.
[c]Dry weight basis.
[d]Hexane extracted by author.
[e]Extraction method unknown.
[f]Expeller extracted by manufacturer.

tions of the different corn brans showed little variation except for samples G, H, and R which showed lower levels of campestanyl ferulate and corresponding increases in sitostanyl ferulate levels. There was an increase in the percentage of *p*-coumarates in the expeller cake; however, the unrefined corn oils, both expeller and hexane-extracted, were similar to the brans. The higher level of esters in the bran duster thrus fraction may be the result of an enrichment of this fraction in aleurone fragments which, since this layer is quite fragile by itself, would not be retained in the bran after duster fraction except as a part of a larger pericarp fragment. If the contribution of the endosperm to this fraction could be corrected for, the yield would be considerably higher. It was interesting that expeller cake had a significant level of esters.



SCHEME 1

The only previous study on the steryl CAD composition of different seed fractions was that of Seitz (1); however, the ester levels of the germ were not determined in that study. The presence of substantial amounts of esters in unrefined corn oils, both expeller and hexane-extracted, and in the cake remaining after expeller extraction, indicates that steryl CAD esters also occur in the germ as corn oil is extracted from the germ fraction of corn. Steryl ferulate esters have been found in corn germ oil (13) but their levels were not reported. Compared to bran and unrefined corn oils, the refined oils, samples S and T, contained only traces of the two major stanyl esters. Refined oils, such as S and T, are processed through several steps (including degumming, caustic refining, bleaching and deodorization), which apparently remove most of the esters. From 50–90% of the γ-oryzanol is removed in refining rice bran oil (7,11). Samples I/W and U/V showed that oils from the same source were similar in content but not identical.

Of particular interest is the effect of the method of extraction on the yield of esters from corn (Table 1). Samples J and K were from the same processor but were extracted by the expeller method for K, and by hexane extraction for J. Moreover, the amount of esters isolated in the latter case was 553% greater. The levels found in expeller oils from samples I and W were similar to those of K, but two other expeller oils (U,V) were considerably lower. The effect of the extraction method showed a similar pattern in rice bran oils (Table 2). No commercial sample of unrefined rice bran oil identified as hexane-extracted was available, but bran extracted by us in the laboratory (N) showed a similar 530% higher level over that of the highest commercial oil examined (M). The other sample (L) had apparently been subject to additional refining steps. There was a marked difference in the levels of two components in the rice bran oils vs. γ-oryzanol and rice bran freshly extracted: cyclobranol was present only in trace amounts in γ-oryzanol and bran; however, its level was markedly higher in the rice bran oils examined. The level of 24-methylenecycloartanyl ferulate in the oils decreased proportionately (Table 2). The apparent health benefits of γ-oryzanol have led to increasing interest examining the steps in rice bran oil refining which affect the levels of γ-oryzanol (11), and in alternate processing modes which would retain these components in the finished oil product (14). The values reported here for rice bran and for the oil extracted from it are similar to the γ-oryzanol levels previously reported for rice bran and oil (8,11). The total ester levels of the two commercial oils were also similar to levels previously reported (7,15). Tables 1 and 2 show that the level of steryl ferulate esters in the oil from rice bran was lower than the level in oil from some types of corn bran; however, because of the much higher oil content of rice bran, the yield of esters on a bran basis would be six to eight times higher.

The effect of rice bran, rice bran oil, and γ-oryzanol on cholesterol metabolism has been the subject of several studies which have recently been reviewed (7). Similar studies seem not to have been carried out on the effect of nonrice steryl CAD esters on cholesterol uptake or serum cholesterol levels. As shown in Table 1, ferulate ester of sitostanol accounts for 65–85% of the ester fraction in oil from corn bran. Sitostanol has been reported to be as effective as sitosterol, at 10-fold lower levels, in lowering serum cholesterol in hypercholesterolemic patients (16), and it was suggested that low-dose sitostanol might be a useful hypolipidemic agent for the treatment of mild hypercholesterolemia. Fatty acid esters of cholesterol and of plant sterols are known to be hydrolyzed in the course of fat digestion (17,18). The effectiveness of plant sterols in lowering blood cholesterol levels is thought to result from their ability to displace free cholesterol from intestinal micelles (18). Rukmini and Raghuram (19) reported that cycloartenol is as effective as γ-oryzanol in lowering serum cholesterol in rats, which would be consistent with the idea that cycloartenyl ferulate is hydrolyzed and that the released sterol has an effect similar to that of the sterol taken up in the diet. Sitostanol has a further advantage in that it is not appreciably taken up into the bloodstream (16,20). The limited solubility of sitostanol in fats, however, has made it difficult to administer it (21), and it is only when plant sterols are suspended in lipids that they can be administered at a dose high enough to be effective (18,21). A recent report has shown that the effectiveness of sitostanol was markedly increased when the sterol was administered as a fatty acid ester with the dietary fat (21). Given the high percentage of sitostanol esters in the steryl CAD ester fraction of corn bran oil and the levels of esters comparable to or higher than the ester levels in

rice bran oil, it would seem that further studies on the effect of these compounds on blood cholesterol levels would be needed. The approaches presented in this paper should prove helpful in screening cereals for sterol esters of cinnamic acid derivatives and in selecting the appropriate corn fraction or oil for the isolation of steryl ferulate and *p*-coumarate esters or for feeding studies with crude preparations.

ACKNOWLEDGMENTS

The author wishes to thank Will Duensing of Lauhoff Grain Co. and Michael Smith of Illinois Cereal Mills, Inc. for providing materials, and John R. Bobell for technical assistance.

REFERENCES

1. Seitz, L.M. (1989) *J. Agric. Food Chem. 37*, 662–667.
2. Warnaar, F. (1984) *Phytochemistry 23*, 1049–1053.
3. Endo, T., Ueno, K., and Inaba, Y. (1968) *Yukagaku 17*, 344–348.
4. Chipley, J.R., and Uraih, N. (1980) *Appl. Environ. Micro. 40*, 352–357.
5. Sinha, K.K., and Singh, P. (1981) *Indian Phytopathology 34*, 530–531.
6. Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S., and Klich, M.A. (1987) *Ann. Rev. Phytopathol. 25*, 249–270.
7. Nicolosi, R.J., Rogers, E.J., Ausman, L.M., and Orthoefer, F.T. (1993) in *Rice Science and Technology* (Marshall, W.E., and Wadsworth, J.I., eds.) pp. 421–437, Marcel Dekker, Inc., New York.
8. Seetharamaiah, G.S., and Prabhakar, J.V. (1986) *J. Food Sci. Tech. 23*, 270–273.
9. Norton, R.A. (1994) *Cereal Chem. 71*, 111–117.
10. Earle, F.R., Curtis, J.J., and Hubbard, J.E. (1946) *Cereal Chem. 23*, 504–511.
11. Yoon, S.H. and Kim, S.K. (1994) *J. Am. Oil Chem. Soc. 71*, 227–229.
12. Shimizu, M., Ohta, G., Kitahara, S-i., Tsunoo, G., and Sasahara, S-i. (1957) *Pharmaceutical Bull. (Tokyo) 5*, 36–39.
13. Tamura, T., Saikaedani, N., and Matsumoto, T. (1958) *Nippon Kagaku Zasshi 79*, 1011–1014.
14. Orthoefer, F. (1994) *INFORM 5*, 548 (abstr).
15. Rogers, E.J., Rice, S.M., Nicolosi, R.J., Carpenter, D.R., McClelland, C.A., and Romanczyk, Jr., L.J. (1993) *J. Am. Oil Chem. Soc. 70*, 301–307.
16. Heinemann, T., Leiss, O., and von Bergmann, K. (1986) *Atherosclerosis 61*, 219–223.
17. Miettinen, T.A., and Siurala, M. (1971) *Z. Klin. Chem. Biochem. 9*, 47–52.
18. Mattson, F.H., Volpenhein, R.A., and Erickson, B.A. (1977) *J. Nutr. 107*, 1139–1146.
19. Rukmini, C., and Raghuram, T.C. (1991) *J. Am. Coll. Nutr. 10*, 593–601.
20. Hassan, A.S., and Rampone, A.J. (1979) *J. Lipid Res. 20*, 646–653.
21. Vanhanen, H.T., Blomqvist, S., Ehnholm, C., Hyvonen, M., Jauhiainen, M., Torstila, I., and Miettinen, T.A. (1993) *J. Lipid Res. 34*,1535–1544.

[Received August 22, 1994, and in revised form January 21, 1995; Revision accepted January 22, 1995]

ANNOUNCEMENTS

1995

June

2nd International Congress of the International Society for the Study of Fatty Acids and Lipids, June 7–10, National Institutes of Health, Bethesda, Maryland, USA. Information: A.P. Simopolous, ISSFAL, 2001 S St., N.W., Suite 530, Washington, DC 20009 USA.

18th Nordic Lipid Symposium, June 18–21, Reykjavik, Iceland. Information: G. Lambertsen, Lipidforum Secretariat, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-325724).

August

36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

November

XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

EDITORIAL

This month, *Lipids* contains a series of papers that originated from an AOCS symposium on isopentenoid metabolism and function. The range of topics runs from overviews of the complexities of this class of compounds in plants and animals, to new thoughts on its importance and pharmacologic manipulation derived from molecular biology approaches. A number of the articles also contain new primary data from the authors, producing a group of contrasting and interrelated articles that we hope will be thought-provoking for our readers. Portions of future issues will occasionally be devoted to thematic works such as these, in order to continue the tradition of *Lipids* being a forum for the latest ideas in lipid research.

As the needs and interests of our readership have evolved over the past twenty-nine years, *Lipids* has responded by altering its format and content. Recently, we have received an increasing number of comments on the format of our references. Titles have never been included in the references of *Lipids* papers, because this was the traditional format for the chemical literature and a major interest at *Lipids* has always been lipid chemistry. These days, however, many of our readers and contributors are from the biomedical disciplines and are used to having access to the titles of referenced papers. This allows readers to quickly decide whether a particular article referred to by our authors would be of interest to them, and has a positive impact on their impression of an article's (and, by extension, a journal's) utility to them. Therefore, we will be adding the titles to the references of articles published in *Lipids,* while keeping all other aspects of the reference format the same. We hope that this addition will be particularly helpful for the review articles that will be published on a regular basis in *Lipids.*

Again, *Lipids* appreciates comments and suggestions from the lipid research community, in addition to your submitted manuscripts!

Howard R. Knapp
Editor-in-Chief

Mechanisms of Free Radical Oxidation of Unsaturated Lipids

Ned A. Porter*, Sarah E. Caldwell and Karen A. Mills
Department of Chemistry, Duke University, Durham, North Carolina 27708

ABSTRACT: The primary products formed from the autoxidation of lipids can be understood based upon a mechanism that involves five different reaction types. These reactions are: reaction of a carbon radical and molecular oxygen, atom transfer of a hydrogen from substrate to the chain carrying peroxyl, fragmentation of the chain carrying peroxyl to give oxygen and a carbon radical, rearrangement of the peroxyl, and cyclization of the peroxyl. The mechanism of these primary reaction steps has been the focus of extensive research over the past fifty years, and the current level of understanding of these transformations is the subject of this review.
Lipids 30, 277–290 (1995).

The study of the spontaneous reaction of molecular oxygen with lipids has been of interest for centuries. Lavoisier (1) was interested in the combustion and the low temperature oxidation of fats and oils, and such reactions are the basis of elemental analysis, a critical development in the birth of the science of organic chemistry. In the past fifty years, interest in the processes responsible for the deterioration of manufactured goods, such as rubber and waxes (2,3), has stimulated the study of autoxidation, the spontaneous free radical reaction of organic compounds with oxygen (4). In more recent years, this process has received renewed attention because of its probable role in destructive biological processes (5). In living systems, autoxidation of lipids, known as lipid peroxidation (6,7), has been implicated in DNA and protein modification, radiation damage, aging and age pigment formation, modification of membrane structure, tumor initiation, and in the deposition of arterial plaque associated with low-density lipoprotein modification (6–13). For these reasons, lipid peroxidation is a subject of continuing interest to the scientific community.

PROPAGATION STEPS IN FREE RADICAL CHAIN OXIDATION

Autoxidation is a free radical chain process consisting of chain initiation, propagation, and termination steps (Fig. 1). The key event in initiation is the formation of a lipid radical, R·. This can occur by thermal or photochemical homolytic cleavage of an RH bond or by hydrogen atom abstraction from R-H by an initiator free radical. The propagation step normally begins with the addition of molecular oxygen to R· and the second step of propagation, the rate-limiting step, is abstraction of a hydrogen atom from RH by peroxyl radical ROO· to generate ROOH and another radical R·.

The rate constant k_p for the rate-limiting step of propagation depends primarily on the strength of the C-H bond being broken. The bond dissociation energy of an allylic hydrogen is approximately 10 kcal/mol greater than that of a bisallylic hydrogen (14). As a result, reasonable rates for the autoxidation of monoenes, such as methyl oleate, can only be achieved at elevated temperatures, while the autoxidation of dienes, such as methyl linoleate, occurs readily at room temperature (Scheme 1).

Propagation steps observed in autoxidation may be more complicated than the simple transfer and additions steps shown in Figure 1. In general, propagation steps observed in free radical chain processes include radical coupling with oxygen, atom or group transfer, fragmentation, rearrangement, and cyclization (Fig. 2). All five of these types of reactions have been observed in the autoxidation process (15).

Molecular oxygen is a ground state triplet having two unpaired electrons; consequently, molecular oxygen is a diradical, and reaction with an organic free radical [Fig. 2(a)] is es-

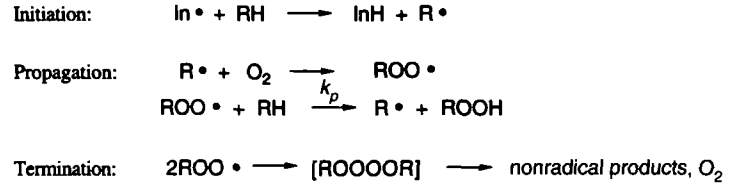
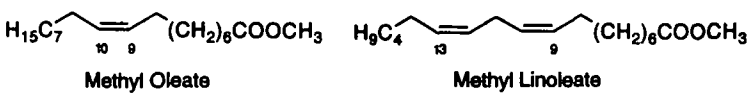


FIG. 1. Chain sequence for free radical autoxidation.

*To whom correspondence should be addressed at the Paul M. Gross Chemical Laboratories, Department of Chemistry, Duke University, Durham, NC 27708.

Abbreviations: GC/MS, gas chromatography/mass spectrometry; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance.

Copyright © 1995 by AOCS Press



SCHEME 1

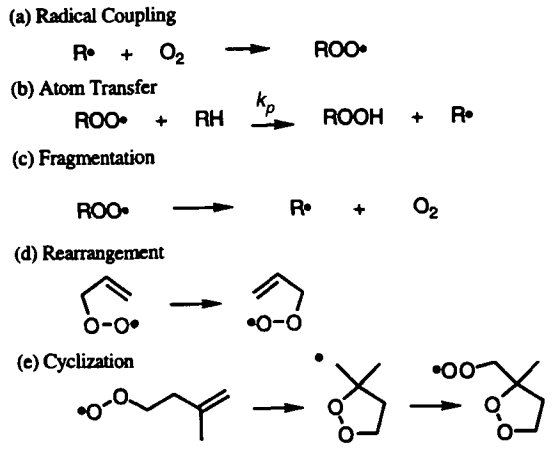


FIG. 2. Propagation reactions in autoxidation.

sentially a radical–radical coupling process (16). Molecular oxygen reacts with the carbon-centered free radical at or near the diffusion controlled rate of approximately 10^9 $M^{-1}s^{-1}$.

Peroxyl radicals abstract hydrogen from organic substrates in a reaction classified as an atom transfer reaction [Fig 2(b)]. At oxygen pressure above 100 torr (17), the rate of oxidation is given by Equation 1 where k_p and k_t are the propagation and termination rate constants, respectively, while R_i is the rate of radical initiation.

$$-dO_2/dt = k_p[\text{R-H}]R_i^{1/2}/2k_t^{1/2} \quad [1]$$

The β-fragmentation reaction of peroxyl radicals, which is the reverse reaction of the oxygen coupling to an organic free radical, is the third type of general process that occurs in the autoxidation propagation sequence [Fig. 2(c)]. This reaction is known to occur when *R* is a stabilized radical such as in the dienylperoxyl radical (see Fig. 14 later in the text) derived from methyl linoleate. Additionally, the first step of the proposed [2,3] allylperoxyl rearrangement mechanism [Fig. 2(d)], has been suggested to be a β-fragmentation of an allylperoxyl to give an allyl radical-dioxygen caged pair.

Peroxyl radicals undergo cyclization reactions, essentially an intramolecular radical addition, to yield five- and six-membered rings [Fig. 2(e)]. Radical cyclization was first proposed to occur in the autoxidation of polyisoprenoids (6). The radical formed in the kinetically favored 5-*exo* cyclization adds a second molecule of oxygen to yield a new peroxyl radical (5). Peroxyl radical cyclization is an important process in the autoxidation of fatty acids or esters having three or more double bonds.

AUTOXIDATION OF MONOENE LIPIDS

Studies of the autoxidation of methyl oleate date back to 1943 in work reported by Farmer and Sutton (18). These workers suggested that the products of oleate oxidation consist of a mixture of allylic hydroperoxides substituted at the C_8 and C_{11} of the eighteen carbon chain. Later, Farmer, Koch and Sutton (19) proposed a free radical chain mechanism involving intermediates **1** and **2** (Fig. 3). They postulated that there would be equal probability for the hydroperoxy group to appear at positions 8, 9, 10, and 11, and for the olefin to remain at the original position (Δ9–10) or to appear at the two adjacent C-C bonds (Δ8–9 and Δ10–11). Thus, it was suggested that autoxidation of methyl oleate yielded four hydroperoxides **3**–**6**. This was supported by work performed by Bickford *et al.* (20) in 1948 and Ross *et al.* (21) in 1946, in which the position of substitution of the hydroperoxy groups was established at C_8, C_9, C_{10}, and C_{11}. The products were confirmed

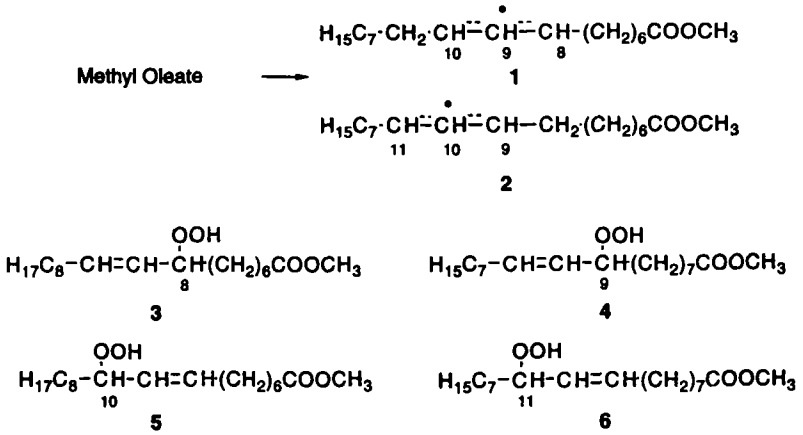


FIG. 3. Oleate product distribution postulated by Farmer, Koch, and Sutton (Ref. 19).

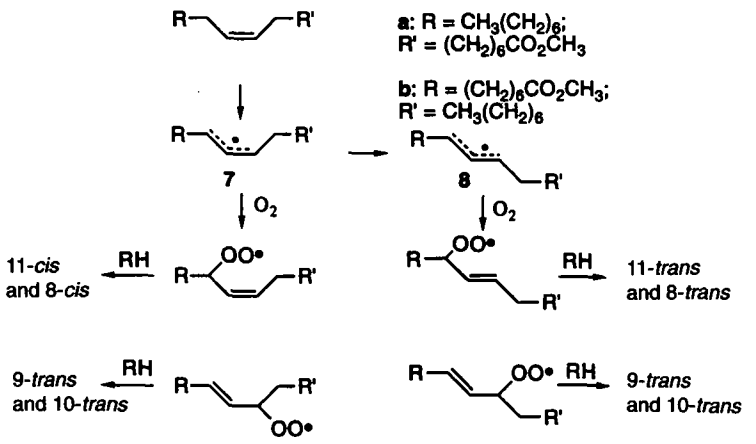


FIG. 4. Proposed methyl oleate autoxidation mechanism, Frankel *et al.* (Ref. 24).

by conversion of the oleate hydroperoxides to the corresponding keto stearic acids.

Prior to 1977, mixtures of oleate allylic hydroperoxides were not fully characterized due to a lack of appropriate analytical techniques (22). That year, Frankel *et al.* (23) reported a structural investigation of methyl oleate autoxidation products using gas chromatography/mass spectrometry (GC/MS). Oleate hydroperoxides were reduced and silylated followed by hydrogenation of the olefin. The resulting trimethylsilyl ethers of the saturated hydroxy esters have good gas chromatographic properties and produce characteristic fragments. This approach provided a direct, reliable method for analysis of regioisomeric oleate hydroperoxides. The 8- and 11-hydroxy isomers were consistently present in higher abundance than the 9- and 10-hydroxy isomers. The difference between these pairs is greatest when the autoxidation was carried out at 40°C, and it decreased when the reaction was run at 80°C.

In 1984, ^{13}C nuclear magnetic resonance (NMR) was used to determine the *cis/trans* geometry of all eight *cis* and *trans* allylic 8-, 9-, 10-, and 11-oleate hydroperoxides (24). The method, along with GC/MS, revealed that autoxidation of methyl oleate occurs symmetrically about the double bond. At 25°C, percentages of the 8-hydroperoxide, 26.4% (*cis/trans* = 14.1:12.3), closely parallel the 11-hydroperoxide, 26.6% (*cis/trans* = 13.7:12.9), and the 9-hydroperoxide, 24.2% (*cis/trans* = 1.1:23.1), is similar to the 10-hydroperoxide, 22.8% (*cis/trans* = 1.1:21.7), in both yield and *cis/trans* ratio.

The distribution of oleate oxidation products was explained by the mechanism outlined in Figure 4. Hydrogen atom abstraction from the 11 position was proposed to lead to delocalized radical **7a** that isomerizes, particularly at elevated temperatures, to give the isomeric allyl radical **8a**. Reaction of the allyl radical **7a** with molecular oxygen was proposed to generate to the 11-*cis* and 9-*trans* oleate hydroperoxides, and reaction of **8a** was proposed to generate the 11-*trans* and 9-*trans* oleate hydroperoxides. Also, hydrogen atom abstraction from carbon 8 was proposed to form delocalized radical **7b** that would isomerize to allyl radical **8b**. Addition of molecular oxygen to **7b** and **8b** was expected to generate the 8-*cis*, 8-*trans* and 10-*trans* oleate hydroperoxides (25).

The direct allyl radical isomerization shown in Figure 4 seems unlikely, since, under normal oxygen pressures, the allyl radical would be trapped with molecular oxygen at the diffusion controlled rate of 10^9 $M^{-1}s^{-1}$, making the allylperoxyl radical the main chain-carrying radical. Because of this, an alternative mechanism was proposed for the formation of products that involves competing peroxyl rearrangement and H-atom abstraction (5). This mechanism, outlined in Figure 5, involves hydrogen atom abstraction at the 8 and 11 positions of methyl oleate to give two allylic radicals **9** and **10**. Due to the symmetry of the oleate autoxidation, only the top portion of Figure 5, with hydrogen atom abstraction from position 11, is discussed here. Addition of O_2 to allyl radical **9** gives rise to two peroxyl radicals, 11-*cis*, **11**, and 9-*trans*, **12.** These peroxyl radicals interconvert *via* a [2,3] allylperoxyl rearrangement. The 11-*trans* peroxyl radical, **13**, is formed by [2,3] allylperoxyl rearrangement of the 9-*trans* peroxyl radical, **12**. Hydroperoxides **14–16** result from these peroxyl radicals undergoing hydrogen atom abstraction from methyl oleate, RH.

Due to the symmetry of the system, similar chemistry occurs for allyl radical **10**, giving hydroperoxides **17–19**. If this mechanism is operative, the product distribution would be dependent upon the hydrogen atom donating ability of the medium. In the presence of a "good" hydrogen atom donor (a compound with a C-H bond of low dissociation energy) the "kinetic" allylperoxyl radicals, **11**, **12**, and their C_8 and C_{10} equivalents, would be trapped prior to [2,3] rearrangement and formation of **16** and **19**. The product distribution would therefore consist of hydroperoxides **14**, **15**, **17**, and **18** with no thermodynamic products **16** and **19** being formed.

The results of a recent study support the mechanism presented in Figure 5 (26). The alcohols that result from reduction of hydroperoxides **14–19** separate by normal-phase high-pressure liquid chromatography (HPLC), and a complete analysis of the products can be achieved using ultraviolet detection at 205 nm. Most methyl oleate or oleic acid obtained commercially is contaminated with linoleate. Linoleate is

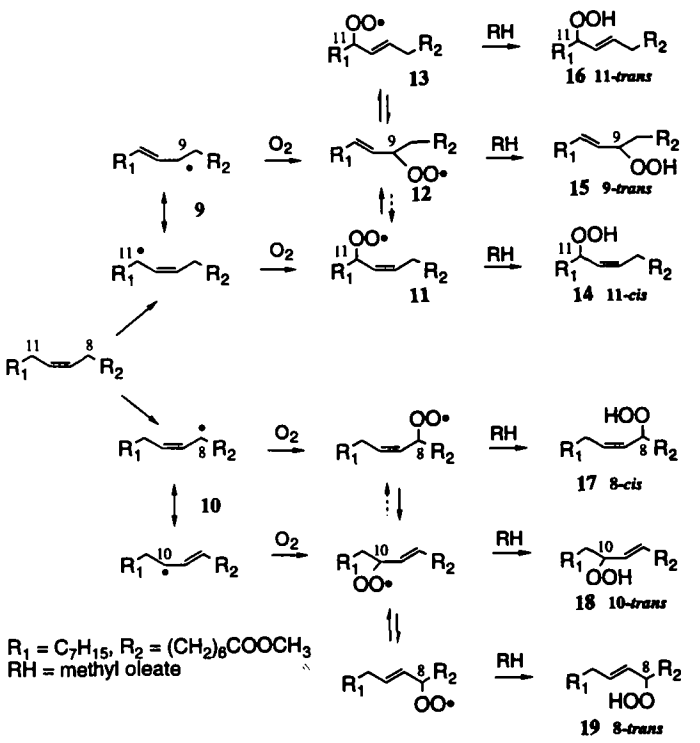


FIG. 5. Proposed mechanism for oleate autoxidation, Porter (Ref. 5).

substantially more oxidizable than oleate, and autoxidation therefore provides substantial quantities of the linoleate hydroperoxides. Due to the excellent chromophore of these conjugated diene hydroperoxides (and alcohols), the linoleate products create a severe problem for analysis of the oleates. In the studies of mechanism reported (26), quantitative data were obtained from oleate samples that had been recycled through autoxidation more than five times. Autoxidation of 1.8 M methyl oleate in benzene at 30°C gives a product distribution as follows: **14**, 11-*cis*, 13%; **15**, 9-*trans*, 21%; **16**, 11-*trans*, 16%; **17**, 8-*cis*, 13%; **18**, 10-*trans*, 21% ; **19**, 8-*trans*, 16%; 10-*cis*, 1%; 9-*cis*, 1%.

If the mechanism in Figure 5 is correct, increasing amounts of "kinetic" 11-*cis* and 9-*trans* hydroperoxides and decreasing amounts of "thermodynamic" 11-*trans* hydroperoxide will form as the concentration of hydrogen atom donors in the medium is increased. If autoxidations are carried out on solutions with starting oleate in the concentration range of 0.3 to 3.0 M, hydroperoxide product distribution is dependent on the concentration of starting oleate. At high starting oleate concentration, more of the "kinetic" products are formed; at low starting oleate concentration, the product mixture contains more "thermodynamic" products.

Courtneidge and Bush (27–29) have demonstrated that *tert*-butyl hydroperoxide-loaded autoxidation reactions are successful in suppressing rearrangements of allylperoxyl radicals, and this approach has been used in oleate autoxidation (26). Hydrogen atoms exchange rapidly between peroxyl radicals. The autoxidation of oleate in the presence of excess *tert*-butyl hydroperoxide, therefore, transfers the chain from oleate peroxyl radicals to *tert*-butyl peroxyl radicals [Equation 2 (Refs. 30–33)].

$$ROO\cdot + (CH_3)_3COOH \xrightarrow{k_p = 2{,}500\ M^{-1}s^{-1},\ 30°C} ROOH + (CH_3)_3COO\cdot \quad [2]$$

Under cooxidation conditions of 4.0 M *tert*-butyl hydroperoxide and 1.8 M oleate (30°C), only the 11-*cis*, 9-*trans*, 10-*trans*, and 8-*cis* oleate hydroperoxides are produced, and no 11- or 8-*trans* hydroperoxides are obtained. The ratio of the 11-*cis*/9-*trans* products is 1:1.2, as is that of the 8-*cis*/10-*trans* compounds. Autoxidations of oleate in the presence of *tert*-butyl hydroperoxide displayed a substantial acceleration compared to oxidations carried out in the absence of the cooxidant. The increase in the rate of oxidation is explained by a reduction in the termination rate of chain carrying *tert*-butyl peroxyl radicals compared to oleate peroxyl radicals (34,35).

The results from the cooxidation of oleate and *tert*-butyl hydroperoxide support the mechanism of oleate autoxidation proposed in Figure 5. According to Figure 5, the initially-formed carbon radical, **9**, has two resonance forms, corresponding to Δ9–10 *cisoid* and Δ10–11 *transoid* geometry. Addition of oxygen leads to the 11-*cis* and 9-*trans* peroxyl radicals, and subsequent hydrogen atom abstraction yields the 11-*cis* and 9-*trans* hydroperoxides **14** and **15**. Similar chemistry after H-atom abstraction at C_8 provides the 8-*cis* and 10-*trans* hydroperoxides. Indeed, these four hydroperoxides are the only products formed in the hydroperoxide-loaded autoxidations. Hydroperoxides are excellent hydrogen atom donors, and the data suggest that, in the presence of hydroperoxide, the product mixture is under kinetic control. The ini-

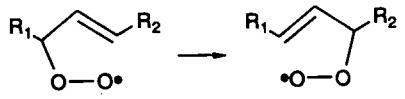


FIG. 6. [2,3] Allylperoxyl rearrangement.

tially-formed peroxyl radicals are directed to the "kinetic" hydroperoxides as a result.

Rearrangements of allyl peroxyls. As shown in Figure 6, allylperoxyl radicals undergo [2,3] rearrangements in which the oxygen atoms, the two component, migrate across the allylic backbone, the three component (6,36–39). A rearrangement of this type is proposed in oleate oxidation to account for the distribution of products formed. Such rearrangements may also be involved in the rearrangement of allylic hydroperoxides. The free radical nature of the hydroperoxide rearrangement is supported by the observation that the rearrangement is catalyzed by free radical initiators or light, and the rearrangement is inhibited by phenolic antioxidants (40). The rearrangement occurs most rapidly in nonpolar aprotic solvents using dilute solutions of hydroperoxide. In concentrated solutions, hydroperoxides form dimers, and hydrogen atom abstraction to form allylperoxyl radicals occurs readily from hydroperoxide monomers but not hydroperoxide dimers (41).

Despite the fact that [2,3] allylperoxyl rearrangements have been known since the late 1950s, the mechanism is still open to debate (6). In 1958, Schenck *et al.* (42,43) reported on the rearrangement of cholesterol hydroperoxides in which the tertiary hydroperoxide 5α-hydroperoxy-3ß-hydroxycholest-6-ene, **20**, slowly rearranges to its secondary allylic isomer, **21**, with complete retention of the α configuration (Fig. 7).

Over twenty years later, Smith (44) showed that compound **21** undergoes a slower epimerization to the 7β-hydroperoxide, **22**. In 1989, Beckwith, Davies, and co-workers (45,46) performed the cholesterol allylperoxyl rearrangement under $(^{18}O)_2$ and observed no ^{18}O incorporation in the 7α-hydroperoxy-3β-hydroxycholest-5-ene, **21**, whereas the epimerization of this product to 7β-hydroperoxy-3β-hydroxycholest-5-ene, **22**, involves 73–83% ^{18}O incorporation. They (45,46) suggested that the rearrangement of **20** to **21** involves a concerted [2,3] allylperoxyl rearrangement, whereas the second rearrangement of **21** to **22** proceeds through a dissociative mechanism.

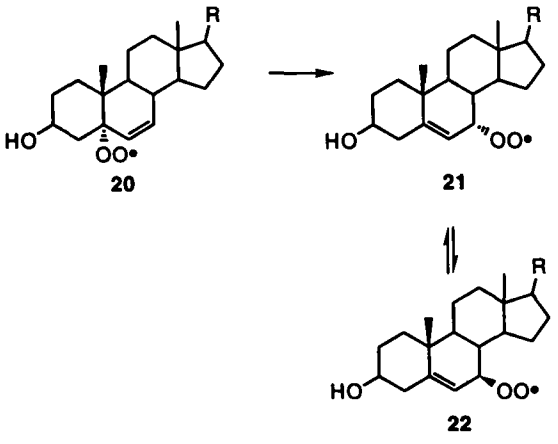


FIG. 7. Cholesterol [2,3] allylperoxyl rearrangement.

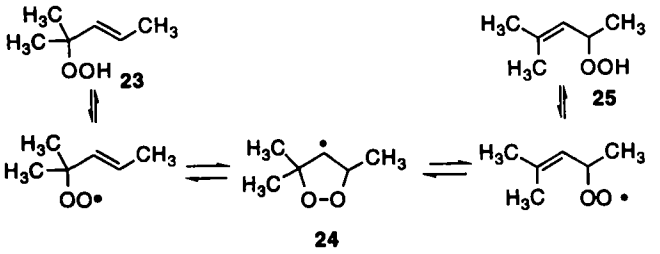


FIG. 8. Cyclization mechanism of [2,3] allylperoxyl rearrangement.

Work on acyclic systems was first reported by Brill (47,48) in 1965. It was observed that either *trans*-2-hydroperoxy-2-methyl-3-pentene, **23**, or its allylic isomer *trans*-2-hydroperoxy-4-methyl-3-pentene, **25**, isomerizes to an equilibrium mixture containing approximately equal quantities of each hydroperoxide **23** and **25** in hexane or carbon tetrachloride at 40°C (Fig. 8). Brill (47,48) concluded that the rearrangement was a free radical intramolecular process occurring *via* a five-membered cyclic intermediate (dioxolanyl radical **24**).

As shown in Figure 9, three mechanisms have been most often suggested for allylperoxyl rearrangement (44–49). The first consists of a stepwise process involving a dioxolanyl radical intermediate like **24**. Another viable mechanism consists of a concerted mechanism involving a five-membered ring peroxide transition state. Finally, an alternate stepwise process has also been suggested in which the peroxyl radical undergoes β-fragmentation to give an allyl radical and molecular O_2. In a follow up step, O_2 recombines at either end of the allyl radical to convert it to the rearranged peroxyl radical or to revert to the starting peroxyl radical.

In later work, Brill (41) provided convincing evidence to call into question the legitimacy of dioxolanyl radical **24** (41). The free radical rearrangement of pinocarvyl hydroperoxide was performed, and there was no evidence for an expected rearrangement of the putative intermediate dioxolanyl radical.

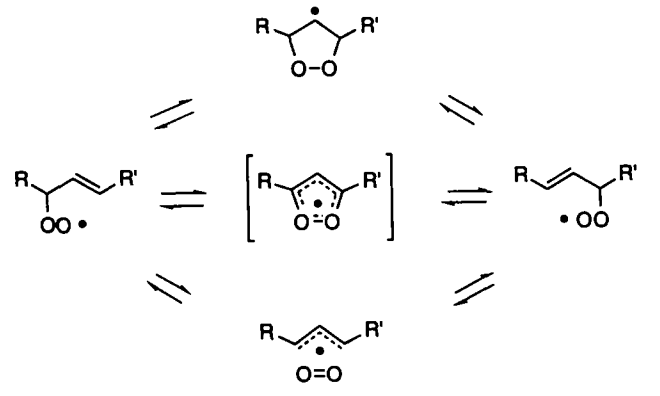


FIG. 9. Possible mechanisms of allylperoxyl rearrangement.

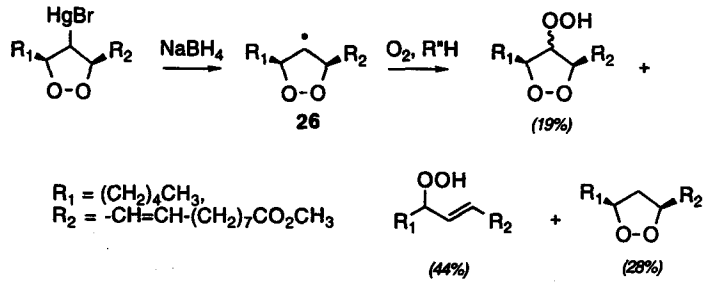


FIG. 10. Oxygen entrapment of the proposed dioxolanyl radical intermediate.

If the dioxolanyl radical is an intermediate along the rearrangement pathway, it would be expected to be trapped to form a cyclic peroxide–hydroperoxide in the presence of oxygen while structural rearrangement would be expected in the absence of oxygen. The data suggest that the dioxolanyl radical is not involved in the [2,3] allylperoxyl rearrangement mechanism.

A later study of an authentic localized dioxolanyl radical demonstrated that it could be trapped with molecular oxygen (49). The postulated intermediate **26** was obtained by the $NaBH_4$ reduction of a mercuric bromide precursor, and the radical was successfully trapped with O_2 to generate a cyclic peroxide–hydroperoxide (Fig. 10). These products are not formed in the rearrangement of the corresponding allylic hydroperoxide. This experiment therefore supports the notion that a dioxolanyl radical is not a discrete intermediate in the rearrangement.

Recent theoretical investigations failed to find the concerted transition state proposed for the rearrangement (50). These calculations were performed at the unrestricted Hartree-Fock and Moeller-Plesset levels using the simple allylperoxyl radical **27** (Fig. 11). Attempts to locate the concerted transition state structure revealed a dioxolanyl radical, **28**, close in energy to the allylperoxyl radical. Radical **28** has a barrier to formation in excess of 40 kcal/mol, which is unreasonably large for a free radical propagation step. The calculations also reveal that β-fragmentation to the allyl radical–dioxygen intermediate, **29**, requires only 22 kcal/mol; therefore, the calculations suggest that the [2,3] allylperoxyl

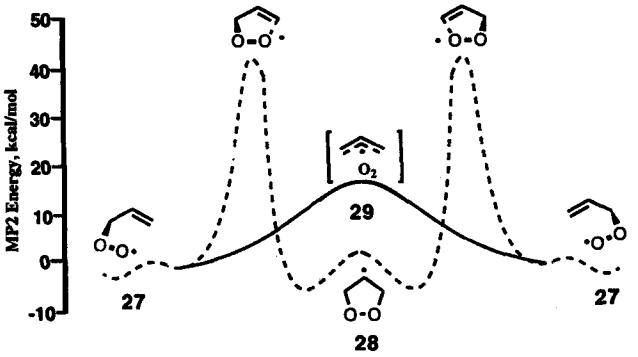


FIG. 11. Theoretical investigations of the allylperoxyl rearrangement.

rearrangement proceeds by this lower energy β-fragmentation process. Hence, the mechanism consistent with experiment and theory involves an allyl radical–triplet dioxygen solvent caged pair, **29** (Fig. 11).

The stereochemical course of the allylperoxyl rearrangement was studied with hydroperoxides derived from oleic acid (51). The rearrangement is highly stereoselective, and the 11-*trans* hydroperoxide rearrangement product derived from the 9-*trans* hydroperoxide has the opposite configuration from the starting hydroperoxide (Fig. 12). As an example of the stereoselectivity of the rearrangement, *(R)*-9-*trans* with an enantiomeric excess >99% rearranges at 22°C to yield a mixture of *(R)*-9-*trans* and *(S)*-11-*trans* with an enantiomeric excess in the latter compound of 97.2% ± 0.5 at the

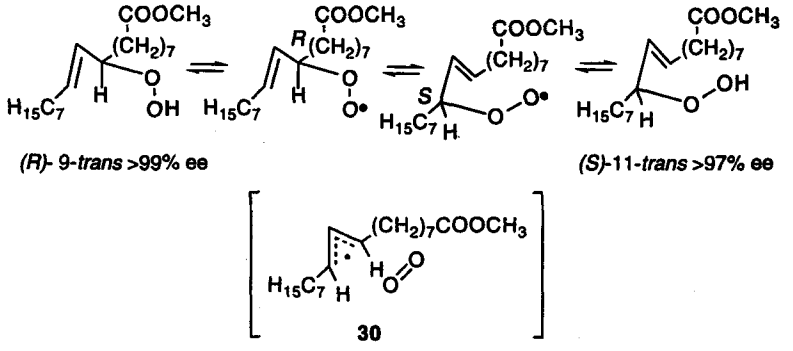


FIG. 12. Stereoselectivity of the *trans* allylperoxyl rearrangement; ee, enantiomeric excess.

time where 9*R*/11*S* = 1.7. The stereoselectivity is shown experimentally to be dependent on temperature with an enantiomeric excess of 97% at 22°C and 94% at 50°C.

The stereoselectivity of the rearrangement is consistent with a concerted mechanism *via* an envelope-like transition state that results in transfer of chirality across the allyl system or with a mechanism involving a very tight allyl–oxygen pair, **30**, which collapses with predominant retention of configuration. One anticipates that study of the rearrangement under an $(^{18}O)_2$ atmosphere would distinguish between the concerted and stepwise mechanisms. The rearrangement proceeds with little incorporation of atmospheric oxygen at room temperature in viscous solvents. At higher temperatures, studies of the rearrangement under oxygen-18 in solvents of different viscosity provide mechanistic insights.

In short, it has been reported that incorporation of labelled oxygen from the atmosphere occurs but that this incorporation is solvent viscosity- and temperature-dependent, as is the stereoselectivity of the rearrangement (52,53). In more viscous solvents and at lower temperatures, less atmospheric oxygen is incorporated in the product of rearrangement, and the rearrangement is more stereoselective. In octadecane at 60°C, for example, the rearrangement of a *trans* alkene derived from methyl oleate gives product with over 96% enantiomeric excess and with incorporation of ~2% atmospheric oxygen. In hexane, substantially more atmospheric oxygen is incorporated, and a corresponding decrease in stereoselectivity is observed.

We conclude that the simplest mechanism for the rearrangement consistent with the experimental results and theoretical investigations involves the allyl–radical dioxygen caged pair **30** that collapses at a diffusion-controlled rate with stereochemical memory. It should be noted that the caged pair might have substantial charge-transfer character. However, a full-fledged allyl–superoxide ion pair seems unlikely in hydrocarbon solvents and would not provide a mechanism for escape that is solvent viscosity-dependent. The results rule out a bridged species as an intermediate in the rearrangement. Allyl radicals that diffuse into solution incorporate $(^{18}O)_2$ with racemization of configuration. This gives evidence of a planar allyl radical intermediate that escapes from the initial solvent cage. The pair collapse (^{16}O) product apparently forms with a high degree of stereoselectivity compared to other known radical pair reactions. The stereochemical-oxygen labeling studies indicate that solvent viscosity affects the partitioning between escape and collapse of the allyl radical–dioxygen caged pair. An increase in solvent viscosity results in a decrease in escape product and a corresponding increase in pair collapse product.

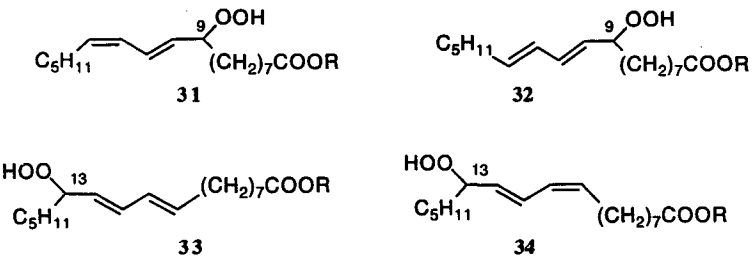
The studies of the [3,2] rearrangement of oleate hydroperoxides (52,53) have a bearing on the mechanism proposed in Figure 5 for oleate autoxidation. Rearrangements of this type are proposed to account for the distribution of products formed in oleate autoxidation, and studies of oleate hydroperoxide rearrangement provide data that are in general agreement with the mechanism proposed in Figure 5. Thus, reaction of the 11-*cis* oleate hydroperoxide, **14**, with free radical initiators gives an allyl peroxyl intermediate **11** that rearranges to the peroxyls **12** and **13.** These peroxyls are ultimately converted to the *trans* hydroperoxides, **15** and **16**. Consistent with the mechanism, *cis* allyl peroxyl radicals rearrange to the related *trans* peroxyl radicals, but the reverse reaction is not observed.

AUTOXIDATION OF DIENE LIPIDS

The autoxidation of methyl linoleate has been extensively studied (6,54,55), and the four major products of this reaction are the hydroperoxides **31–34** (56–58). These conjugated diene hydroperoxides result from oxygen addition at either the 9 or the 13 position of the eighteen carbon chain. Two of the products have *cis, trans* geometry in the conjugated diene unit while the other two display *trans, trans* geometry (Scheme 2).

A mechanism for the formation of the four major products (54–58) of methyl linoleate autoxidation has been elucidated and is outlined in Figure 13. In the first step, hydrogen atom abstraction from C-11 of methyl linoleate leads to the pentadienyl radical **35**. Subsequent oxygen addition to **35** generates one of two *trans, cis* peroxyl radicals, **36** or **37**, either of which has three possible options. Unlike the analogous transformation catalyzed by lipoxygenase enzymes (59), both C-11 hydrogens are removed, and oxygen reacts equally on both faces of the intermediate pentadienyl. Considering just the 13-substituted radical **36**, hydrogen atom abstraction from a donor such as another linoleate generates 13-*cis*, *trans* hydroperoxide **34**, which is the kinetic product.

The other two options involve β-fragmentation of the intermediate peroxyl radical. Nonproductive β-fragmentation



SCHEME 2

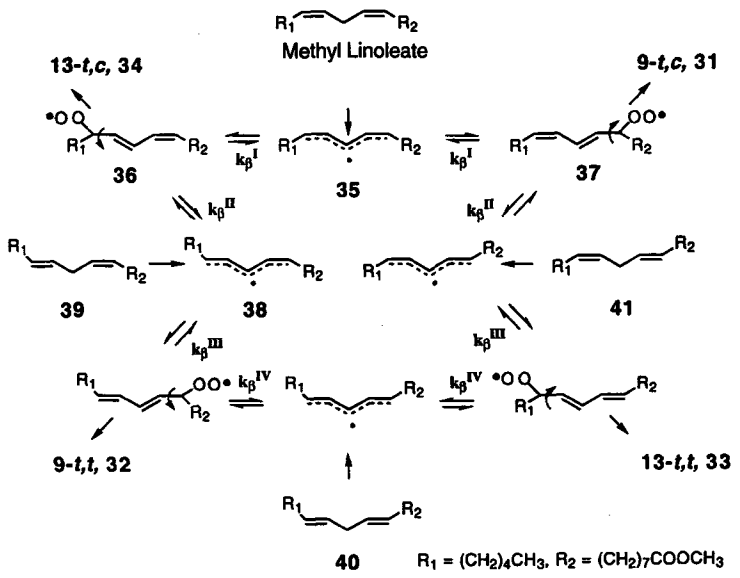


FIG. 13. Proposed mechanism of the autoxidation of octadecadienoates.

gives the original pentadienyl radical **35**. Alternatively, if bond rotation about C12–C13 precedes β-fragmentation, then a new pentadienyl radical **38** is formed.

This radical has *trans* rather than *cis* geometry about the partial double bond Δ-12,13. Oxygen addition at C-9 then leads to a peroxyl radical that gives the 9-*trans*, *trans* hydroperoxide **32**, the thermodynamic product. As can be seen from Figure 13, the same processes can occur with the initially formed 9-substituted peroxyl radical **37.** Autoxidation product ratios are therefore determined by the competition between hydrogen atom abstraction to give the *cis, trans* kinetic products and β-fragmentation to give the *trans, trans* thermodynamic products.

In-depth studies by Chan *et al.* (54) have established that the rearrangement of dienylperoxyl radicals derived from linoleate proceeds by a β-fragmentation mechanism. This was determined through investigations of the thermal rearrangement of the diene hydroperoxide **34** derived from methyl linoleate using an ^{18}O label (Fig. 14). It was demonstrated that ^{18}O-labeled dienyl hydroperoxides undergo rearrangement with incorporation of atmospheric oxygen into the rearrangement products **36** and **37**, giving evidence in support of a β-fragmentation mechanism involving pentadienyl radical intermediate **38**. The rearrangement has been studied in viscous solvents, such as heptadecane, and the products show complete atmospheric oxygen incorporation, unlike the oleates where a cage recombination mechanism has been sug-

TABLE 1
Product Distribution in the Autoxidation of 0.24 M Methyl Linoleate and 39–41[a]

CHD(M)	Dienoate	13-*t,c*	13-*t,t*	9-*t,c*	9-*t,t*
0	39	0.10	0.41	0.05	0.44
Thermodynamic	41	0.06	0.44	0.09	0.41
conditions	40	0.06	0.45	0.05	0.44
	ML	0.11	0.40	0.10	0.39
3.0	39	0.55	0.09	0.03	0.33
Kinetic	41	0.06	0.32	0.54	0.08
conditions	40	0.02	0.49	0.02	0.47
	ML	0.43	0.08	0.41	0.08

[a]CHD, 1,4-cyclohexadiene; ML, methyl linoleate; 13-*t,c*, methyl 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoate; 13-*t,t*, methyl 13-hydroperoxy-*trans*-11,*trans*-9-octadecadienoate; 9-*t,c*, methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate; 9-*t,t*, methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate.

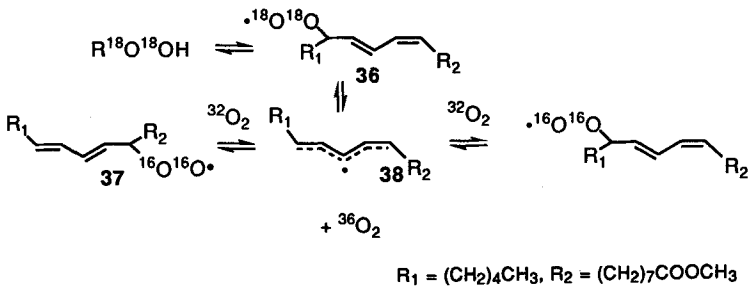


FIG. 14. Proposed mechanism of the dienylperoxyl rearrangement.

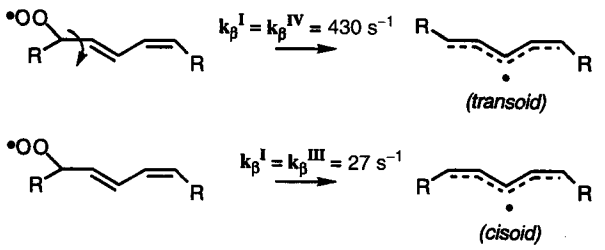


FIG. 15. Dienylperoxyl fragmentation rate constants.

gested (52,53,56,60; Caldwell, S.E., and Porter, N.A., submitted for publication).

As shown in Figure 13, the autoxidation of methyl linoleate and the three isomeric methyl 9,12-octadecadienoates, **39–41,** has been investigated (56,61,62). In the absence of cyclohexadiene (an excellent hydrogen atom donor), the product distribution of diene hydroperoxides **31–34** is similar for each diene precursor, with the thermodynamic *trans, trans* products dominating the product mixture (Table 1). At higher concentrations of cyclohexadiene (i.e., 3.0 M), the product distribution changes to reflect the stereochemistry of the starting diene with the kinetic product distribution being obtained.

A steady-state successive approximation computer analysis was conducted to determine the fragmentation rate constants, k_β^I, k_β^{II}, k_β^{III}, and k_β^{IV} (56,61,62). This approach allows the product distribution to be calculated for each of the dienoate precursors as a function of cyclohexadiene concentration for various values of $k_\beta^{I–IV}$. Through this analysis, it was determined that $k_\beta^I = k_\beta^{III} = 27\ s^{-1}$ and $k_\beta^{II} = k_\beta^{IV} = 430\ s^{-1}$. These rate constants reveal that β-fragmentation of dienylperoxyl radicals leading to *transoid* centers occurs sixteen times faster than fragmentations of peroxyl radicals leading to *cisoid* centers (Fig. 15).

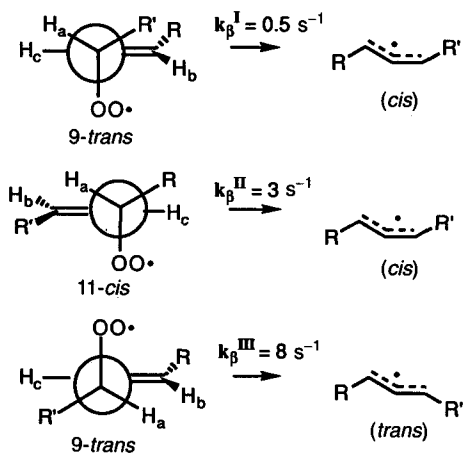
At high concentrations of cyclohexadiene, the product distribution reflects the initial partitioning of oxygen to the ends of the pentadienyl radical. Reaction at the *transoid* end of the pentadienyl radical is twofold that of reaction at the *cisoid* end of the radical. These results are consistent with electron paramagnetic studies that suggest a higher spin density at *transoid* pentadienyl termini (63).

It is of interest to compare the fragmentation rate constants for linoleate with those determined for oleate. The oleate β-fragmentation rate constants are shown in Figure 16 with the allylperoxyl radicals represented as Newman projections. Three different fragmentation reactions occur in the oleate system: (i) fragmentation of the 9- (or 10-) *trans* allylperoxyl radical to generate the *cisoid* allyl radical (k_β^I); (ii) fragmentation of the 11- (or 8-) *cis* allylperoxyl radical to generate a cisoid allyl radical (k_β^{II}); (iii) fragmentation of the 9- (or 10-) *trans* allylperoxyl radical to generate the *transoid* allyl radical (k_β^{III}).

β-Fragmentation of the 9-*trans* allylperoxyl radical can generate either a *transoid* or *cisoid* radical. β-Fragmentation of the 9-*trans* allylperoxyl radical leading to a *transoid* allyl radical is sixteen times faster than β-fragmentation to a *cisoid* allyl radical (8 vs. 0.5 s^{-1}). On the other hand, fragmentation of a *cis* allyl peroxyl radical generates only a *cisoid* allyl radical, and the rate constant for this process is 3 s^{-1}.

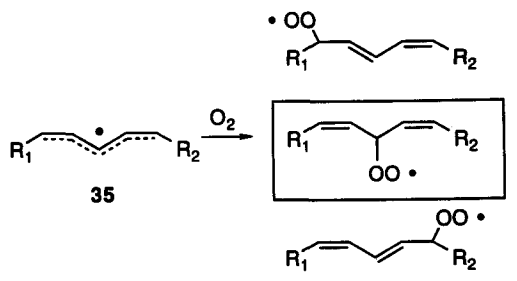
In the linoleate system studied earlier, fragmentation to a *transoid* terminus was also found to occur sixteen times faster than fragmentation to the *cisoid* terminus. Substantial allylic 1–3 strain apparently attends fragmentation processes leading to *cisoid* allyl radicals as the sp^3 hybridized carbon bearing the peroxyl oxygen is converted to an sp^2 hybridized atom of the delocalized carbon radical. It has been suggested that this allylic strain (64) accounts for the rate differences observed for the different fragmentation processes. The peroxyl fragmentation rate constants for oleate are about twenty to fifty times smaller than the corresponding rate constants for fragmentation of linoleate peroxyl radicals. Since product oleate allyl radicals are less stabilized than the linoleate pentadienyl radicals, propagation and fragmentation rate constants in the oleate system are therefore smaller than for linoleate.

One question that arises in the mechanism of linoleate autoxidation is that bisallylic hydroperoxides resulting from oxygen addition to the central carbon of pentadienyl radicals (such as **35**) are not normally observed in autoxidation reactions. An exhaustive analysis of methyl linoleate autoxidation products failed to reveal the presence of a bisallylic prod-



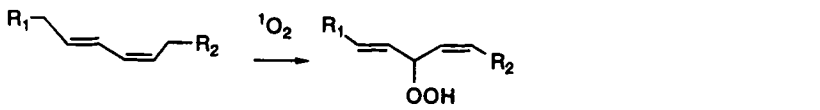
R = C_7H_{15}; R' = $(CH_2)_7CO_2CH_3$

FIG. 16. Fragmentation rate constants for oleate peroxyls.



$R_1 = (CH_2)_4CH_3$, $R_2 = (CH_2)_7COOCH_3$

SCHEME 3



42 43 $R_1 = (CH_2)_4CH_3$, $R_2 = (CH_2)_7COOCH_3$

SCHEME 4

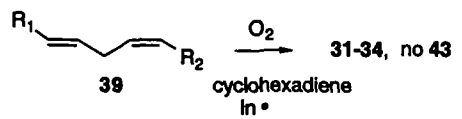
uct, although minor products resulting from abstraction of a singly allylic hydrogen atom at C-8 or C-14 of methyl linoleate were detected (Scheme 3) (65).

The failure to detect bisallylic products is puzzling, because the central carbon has the highest odd electron density in the pentadienyl radical (14). It has been suggested that oxygen attack at the central carbon does occur, but subsequent rearrangement to conjugated diene products (*via* β-fragmentation or [2,3] rearrangement) is rapid (6). This hypothesis was tested by means of a study of the radical rearrangement of an independently generated bisallylic hydroperoxide.

The bisallylic hydroperoxide **43** was prepared by singlet oxygenation of the diene **42** (Scheme 4). The hydroperoxide could be separated and purified from the complex mixture of products resulting from the singlet oxygen reaction. The bisallylic compound, **43**, itself proved to be surprisingly stable, even when concentrated samples were stored at room temperature.

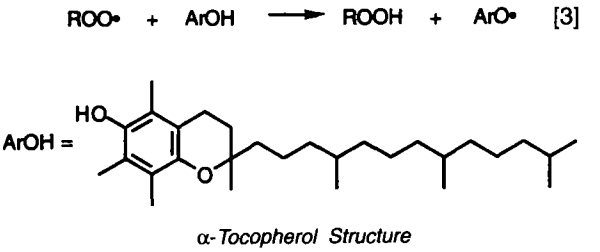
Since the *cis*-9,*trans*-2-bisallylic 11-bisallylic hydroperoxide **43** has been characterized, attempts were made to determine if it is present in the "kinetic" product mixture formed by autoxidation of the appropriate linoleate isomer, **39**. Autoxidation of **39** was carried out at 30 °C in benzene or 1.4 M cyclohexadiene in benzene, initiated by di-*t*-butyl hyponitrite. Autoxidation products were purified by HPLC and analyzed by ^{1}H NMR and HPLC/MS. As expected, the major products of the reaction were the conjugated dienyl hydroperoxides **31–34.** Also detected were minor products resulting from abstraction of a singly allylic hydrogen at C-8 or C-14. However, there was no evidence of the bisallylic product. The results obtained in these autoxidation experiments are therefore consistent with the hypothesis that bisallylic products are not formed as products of lipid autoxidation because oxygen addition to the central carbon of the pentadienyl radical does not occur (Scheme 5) (Caldwell, S.E., and Porter, N.A., unpublished results).

Tocopherol mediated autoxidation. Phenolic antioxidants react with peroxyl radicals to generate a hydroperoxide product and relatively unreactive phenoxyl radical (Refs. 66–70;



$R_1 = (CH_2)_4CH_3$, $R_2 = (CH_2)_7COOCH_3$

SCHEME 5



Equation 3). The most effective chain-breaking antioxidant among the phenolic antioxidants is α-tocopherol, the biologically active component of vitamin E (71). α-Tocopherol, present in cell membranes and edible oils, protects polyunsaturated lipids and fatty acids from peroxidation.

It is generally accepted (67–71) that α-tocopherol inhibits lipid peroxidation by hydrogen atom abstraction from the OH group by a peroxyl radical, LOO• (Eq. 4 in Fig. 17). Hydrogen atom abstraction occurs with a rate constant of $k_1 = 10^6$ $M^{-1}s^{-1}$, about four orders of magnitude faster than the propagation rates for hydrogen atom transfer from hydrocarbons to peroxyl radicals. The products of this H-atom transfer are a tocopherol radical, Toc• and a lipid hydroperoxide. The tocopherol radical can subsequently undergo chain termination reactions with peroxyl radicals in solution (Ref. 72; Eq. 5 in Fig. 17). Reactions 4 and 5 intercept and destroy the chain carrying peroxyl radicals, explaining the antioxidant effect of α-tocopherol.

Several research groups have demonstrated that α-tocopherol in high concentration acts as a prooxidant (73,74) during the autoxidation of polyunsaturated fatty acids by trapping out the kinetic peroxyl radicals and, as a result, altering the stereochemistry of the hydroperoxide products formed (75,76). It is proposed that the tocopherol radicals participate in the prooxidant effect through hydrogen atom abstraction (Eq. 6 in Fig. 17) and the chain-transfer reaction [Eq. 7 in Fig. 17). The rate constant k_{-1} was calculated to be approximately seven orders of magnitude less than k_1, the reverse of Equa-

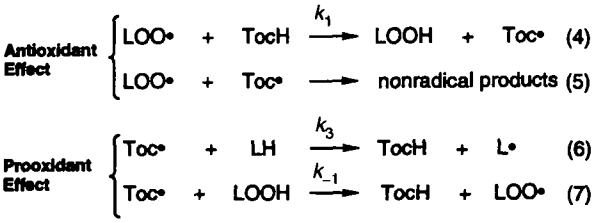
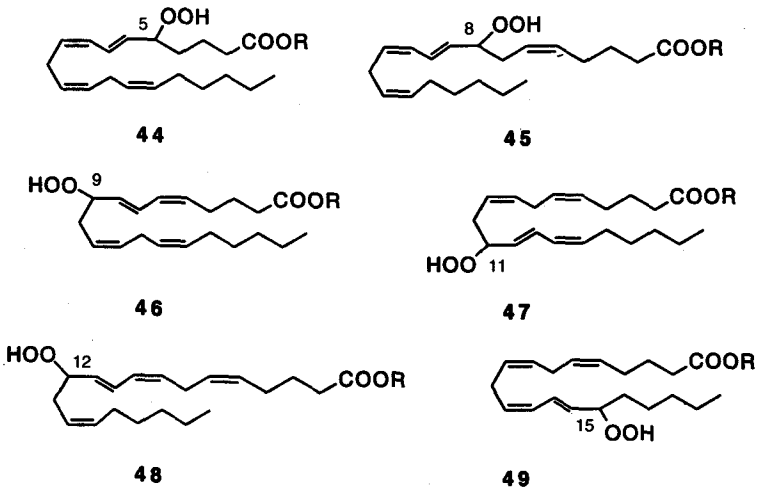


FIG. 17. Antioxidant/prooxidant properties of α-tocopherol.



SCHEME 6

tion 4 (in Fig. 17). Reaction 6 generates another lipid free radical to propagate the chain explaining the ability of α-tocopherol to act as a prooxidant.

The rate constant k_3 was determined to be 1.8×10^{-2} $M^{-1}s^{-1}$ for hydrogen atom abstraction from methyl linoleate by the tocopherol radical (77). This reaction generates a linoleate pentadienyl radical, thus propagating the chain. Autoxidation of methyl linoleate in the presence of the excellent hydrogen atom donor α-tocopherol has been shown to change the product stereochemistry so that the kinetic product distribution is obtained. The ratio of the kinetic *trans, cis* product to the thermodynamic *trans, trans* product is >75:1 in the presence of α-tocopherol and 0.2:1 in the absence of α-tocopherol under otherwise comparable conditions. It is notable that phenols, which have historically been thought to prevent autoxidations, actually provide the kinetic product distribution for linoleate in a phenoxy-mediated reaction.

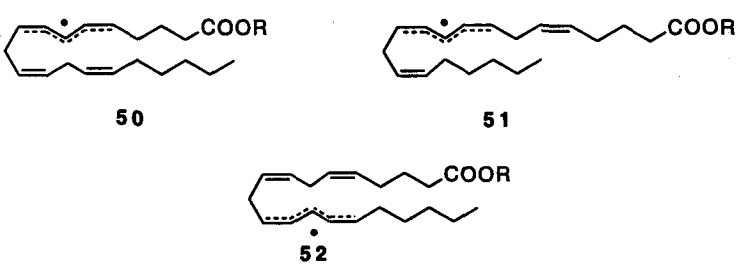
POLYENE AUTOXIDATION

Arachidonic (20:4) and linolenic acid (18:3) autoxidation have been investigated extensively as have pentaenoic and hexaenoic fatty acids (57,78). The free radical initiated oxygenation reaction of arachidonate illustrates the types of products that are generated in polyene autoxidation (57,79–81). The product mixtures in the autoxidation of this acid or its esters are extremely complex. Six simple hydroperoxide products, **44–49**, have been isolated, and these compounds all have *trans-cis* diene geometry. The alcohols formed from these hydroperoxides by reduction can be separated by HPLC, and this allows for extensive investigation of the mechanism of autoxidation (80,81). Smaller amounts of *trans-trans* diene products can be isolated, particularly the 15-*trans-trans* compound (Scheme 6).

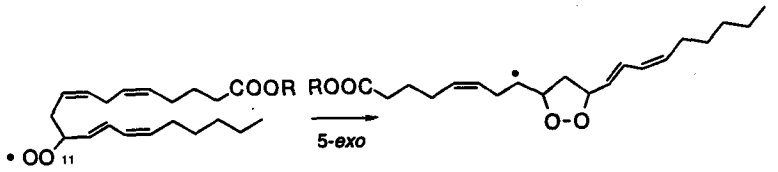
Cyclic peroxide products have also been isolated in the autoxidation of arachidonate and other triene or tetraene fatty acids. To simplify the product mixture of cyclic peroxide products, specific hydroperoxides have been used as precursors to particular peroxyl radicals. For example, O'Connor, Mihelich, and Coleman (82) have investigated the cyclization reactions of the 9-hydroperoxide from γ-linolenate, and they found several cyclic peroxide compounds and proved the structure of these compounds.

For arachidonate, there are three centers at C-7, C-10, and C-13 that have carbons flanked by two double bonds. These carbons are analogous to the C-11 carbon of linoleate and are readily attacked by chain-carrying peroxyl radicals. Thus three pentadienyl radicals are formed as intermediates in arachidonate autoxidation, and these three pentadienyl radicals, **50–52**, all have *cis-trans-trans-cis* geometry by analogy to the pentadienyl radical formed from autoxidation of linoleate.

Oxygen addition occurs at either end of the three pentadi-



SCHEME 7



SCHEME 8

enyl radicals giving six peroxyl free radicals having *trans-cis* diene geometry. Hydrogen atom abstraction by these peroxyl radicals gives the six primary *trans-cis* diene products, **44–49** (Scheme 7).

Scission reactions of these peroxyl radicals are also possible, as is the case for linoleate peroxyl radicals. This scission reaction is a unimolecular process, but radical cyclization, another unimolecular pathway, apparently occurs with a faster rate constant. Consequently, few *trans-trans* products, which are formed by a fragmentation readdition pathway, are observed in arachidonate oxidation. Four of the six peroxyl radicals have isolated double bonds placed such that a 5-*exo* cyclization can occur. These four peroxyl radicals have oxygen substitution at positions C-8, C-9, C-11, and C-12 on the carbon chain. The 5-*exo* cyclizations are relatively facile (83,84) occurring with rate constants on the order of $k_c \sim 10^3\ s^{-1}$ (57). The peroxyl radicals substituted at positions C-5 and C-15 have no such double bond and no cyclization products are observed from these radicals (Scheme 8).

Since four different peroxyl radicals can give cyclic products and since multiple products are possible after cyclization, *vide infra*, many different products form in the autoxidation of arachidonate. Hydroperoxides serve as precursors to specific peroxyl radicals as described earlier, and the chemistry of peroxyl radicals generated in this manner is easier to study than is the case when random autoxidation is used. From one peroxyl radical, several products are formed other than the simple hydroperoxide. Monocyclic peroxides, bicyclic peroxides (85,86) and epoxy alcohols, all formed as several stereoisomers, give rise to literally hundreds of different products in the autoxidation of arachidonates. Other polyene structures may give more or less complicated product mixtures, depending on the number of double bonds in the system. At least three double bonds are required for radical cyclization pathways to exist, and the more double bonds present, the more complex will be the mixture of products.

Peroxyl radical cyclization has been discussed extensively elsewhere (5,6), and products formed from such cyclization of lipid radicals have been isolated from natural sources. Indeed, such cyclization products formed from lipid peroxidation (isoprostanes) may be natural markers of *in vivo* lipid peroxidation. We note also that peroxyl radical cyclization has been used to provide information about the [3,2] allylperoxyl rearrangement. Davies and Davison (46) performed kinetic studies on the [2,3] allylperoxyl rearrangement and found that the rearrangement occurs faster in cyclic as opposed to acyclic systems, but they were not able to quantify the rearrangement rates (46). Using the *tert*-butyl hydroperoxide loaded method, Courtneidge has estimated the rate constant for the [2,3] allylperoxyl rearrangement (Fig. 18). In this study, Courtneidge and Bush (27–29) converted the tertiary allylic hydroperoxide **53**, under azo-*bis*-isobutyryl nitrile initiation, to hydroperoxy cyclic peroxides: hydroperoxydioxane **56** (59%) and hydroperoxydioxolane **57** (41%). Applying the "radical clock" concept, Courtneidge's results, along with the estimate for 5-*exo* peroxyl radical closure of approximately $8 \times 10^2\ s^{-1}$ at 30°C (57), suggest that the 6-*exo* cyclization of **54** to **57** and the [2,3] allylperoxyl rearrangement of **54** to **55** have rate constants of approximately $10\ s^{-1}$ at 30°C.

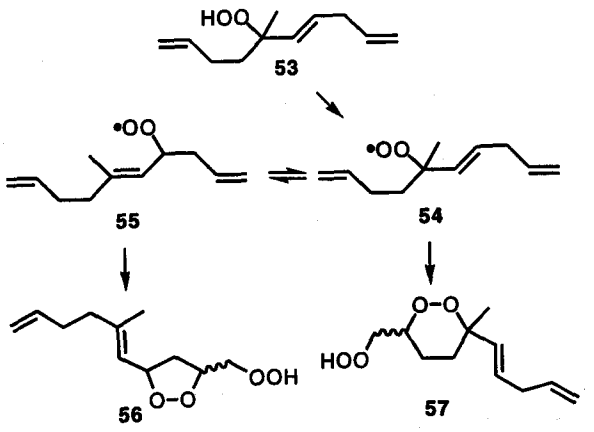


FIG. 18. Free radical initiated conversion of allylic hydroperoxide.

CONCLUSIONS AND PROSPECTS

This review has focused on the primary reactions that occur in the autoxidation of unsaturated fatty acids and esters. The reaction types that are observed in these fundamental transformations are relatively well understood. Hydrogen atom transfer to generate carbon free radicals from the lipid substrate, oxygen coupling to those radicals, peroxyl radical fragmentation and cyclization, and peroxyl radical hydrogen atom abstraction are the important reaction types that have been established in lipid peroxidation. While this review has dealt principally with unsaturated fatty acids and esters, similar mechanisms apply to the autoxidation of other unsaturated natural products, such as steroids (see Refs. 44–46).

Reaction product mixtures of autoxidized lipids are usually very complicated because many products can be formed as a result of these few important propagation reactions. Furthermore, the products of autoxidation, hydroperoxides and cyclic peroxides are themselves very reactive species, and

secondary products form from the decomposition of the primary peroxide oxidation products. The nature of these secondary products and the mechanisms of their formation are beyond the scope of this review.

Recent lipid autoxidation research efforts have focused on peroxidation in aggregates, including particles such as low-density lipoproteins (12,13,87–92). The importance of tocopherol mediated autoxidation and particle size on the course of autoxidation in aggregates has recently been the subject of intensive investigation. These factors can have dramatic consequences for the kinetics of autoxidation (93–97) and are presumably important not only for lipoproteins but also in the autoxidation of aggregates in foodstuffs or in the environment. The unraveling of the dynamics of such processes and how they affect the kinetics and products of autoxidation of these aggregate lipid forms should provide a new understanding of this age-old phenomenon.

REFERENCES

1. Lavoisier, A.L. (1789) *Elements of Chemistry* (translated by Robert Kerr) pp. 107–108, Dover Publications, 1965, New York.
2. Mayo, F.R. (1986) *J. Chem. Ed. 63*, 97–98
3. Walling, C. (1986) *J. Chem. Ed. 63*, 99–101.
4. Walling, C. (1987) *Chem. Br., 23*, 769–770.
5. Porter, N.A. (1986) *Acc. Chem. Res. 19*, 262–268.
6. Porter, N.A. (1990) *Membrane Lipid Oxidation* (Vigo-Pelfrey, C., ed.) Vol. I, pp. 33–62, CRC Press, Boca Raton.
7. Marnett, L.J. (1987) in *Anticarcinogenesis and Radiation Protection* (Cerutti, P.A., Nygard, O.F., and Simic, M.G., eds.) pp. 71–80, Plenum Press, New York.
8. Gershon, D. (1988) in *Reactive Oxygen Species in Chemistry, Biology, and Medicine* (Quintanilla, A., ed.) pp. 211–219, Plenum Press, New York.
9. Cheeseman, K.H., and Slater, T.F. (1993) *British Med. Bull. 9*, 481–493.
10. Gardner, H.W. (1983) in *Xenobiotics in Foods and Feeds, 234* (Finley, J.W., and Schwass, D.W., eds.) pp. 63–98, American Chemical Society Symposium Series, Washington, D.C.
11. Porter, N.A. (1985) in *Chemical Changes in Food During Processing* (Richardson, T., and Finley, J.W., eds.) pp. 73–78, AVI Publishing Co., Inc., Westport.
12. Hessler, J.R., Morel, D.W., Lewis, L.J., and Chisholm, G.M. (1983) *Arteriosclerosis 3*, 215–222.
13. Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., and Steinberg, D. (1984) *Proc. Natl. Acad. Sci. USA 81*, 3883–3887.
14. Davies, A.G., Griller, D., Ingold, K.U., Lindsay, D.A., and Walton, J.C. (1981) *J. Chem. Soc. Perkin Trans. II*, 633–641.
15. Howard, J.A. (1973) in *Free Radicals* (Kochi, J.K., ed.) Vol. II, pp. 15–31, John Wiley and Sons, New York.
16. Nonhebel, D.C., and Walton, J.C. (1984) in *Free Radical Chemistry*, pp. 393–417, Cambridge University Press, London.
17. Howard, J.A. (1972) in *Advances in Free Radical Chemistry,* Vol. IV, pp. 75–138, Academic Press, New York.
18. Farmer, E.H., and Sutton, D.A. (1943) *J. Chem. Soc.*, 119–122.
19. Farmer, E.H., Koch, H.P., and Sutton, D.A. (1943) *J. Chem. Soc.*, 541–547.
20. Bickford, W.G., Fisher, G.S., Kyame, L., and Swift, C.E. (1948) *J. Am. Oil Chem. Soc. 24*, 254–257.
21. Ross, J., Gebhart, A.I., and Gerecht, J.F. (1946) *J. Am. Chem. Soc. 68*, 1373–1376.
22. Frankel, E.N. (1980) in *Autoxidation in Food and Biological Systems* (Simic, M., and Karel, M., eds.) pp. 141–183, Plenum Press, New York.
23. Frankel, E.N., Neff, W.E., and Rohwedder, W.K. (1977) *Lipids 12*, 901–907.
24. Frankel, E.N., Garwood, R.F., Khambay, B.P., Moss, G.P., and Weedon, B.C. (1984) *J. Chem. Soc. Perkin Trans. I*, 2233–2240.
25. Pritzkow, W., Radeolia, R., and Schmidt-Renner, W. (1979) *J. Prakt. Chem. 321*, 813–826.
26. Porter, N.A., Mills, K.A., and Carter, R.L. (1994) *J. Am. Chem. Soc. 116*, 6690–6696.
27. Courtneidge, J.L., and Bush, M. (1992) *J. Chem. Soc. Perkin Trans. I*, 1531–1538.
28. Courtneidge, J.L., Bush, M., and Loh, L.-S. (1992) *J. Chem. Soc. Perkin Trans. I*, 1539–1548.
29. Courtneidge, J.L. (1992) *J. Chem. Soc. Chem. Commun.*, 1270–1272.
30. Howard, J.A., Schwalm, W.J., and Ingold, K.U. (1968) *Adv. Chem. Ser. 75*, 6–23.
31. Middleton, B.S., and Ingold, K.U. (1967) *Can. J. Chem. 45*, 191–194.
32. Chenier, J.H., and Howard, J.A. (1975) *Can. J. Chem. 53*, 623–627
33. Howard, J.A. (1984) *Israel J. Chem. 24*, 33–37.
34. Thomas, J.R., and Tolman, C.A. (1962) *J. Am. Chem. Soc. 84*, 2079–2080.
35. Thomas, J.R. (1967) *J. Am. Chem. Soc. 84*, 4872–4875.
36. Saebo, S., Beckwith, A.L.J., and Radom, L. (1984) *J. Am. Chem. Soc. 106*, 5119–1522.
37. Lindsay, D.A., Lusztyk, J., and Ingold, K.U. (1984) *J. Am. Chem. Soc. 106*, 7087–7093.
38. Barclay, L.R.C., Lusztyk, J., and Ingold, K.U. (1984) *J. Am. Chem. Soc. 106*, 1793–1796.
39. Crich, D., and Filzen, G.F. (1993) *Tetrahedron Lett. 34*, 3225–3229.
40. Frimer, A.A. (1979) *Chem. Rev. 79*, 359–387.
41. Brill, W.F. (1984) *J. Chem. Soc. Perkin Trans. II*, 621–627.
42. Schenck, G.O. (1957) *Angew. Chem. 69*, 579–599.
43. Schenck, G.O., Neumuller, O.A., and Eisfeld, K.C. (1958) *Angew. Chem. 70*, p. 595.
44. Smith, L.L. (1981) *Cholesterol Autoxidation*, Plenum Press, New York.
45. Beckwith, A.L., Davies, A.G., Davison, I.G., Macoll, A., and Mruzek, M.H. (1989) *J. Chem. Soc. Perkin Trans. II*, 815–824.
46. Davies, A.G., and Davison, I.G. (1989) *J. Chem. Soc. Perkin Trans. II*, 825–830.
47. Brill, W.F. (1965) *J. Am. Chem. Soc. 87*, 3286–3287.
48. Brill, W.F. (1968) *Adv. Chem. Ser. 75*, 93–101.
49. Porter, N.A., and Zuraw, P. (1985) *J. Chem. Soc. Chem. Commun.*, 1472–1473.
50. Boyd, S.L., Boyd, R.J., Shi, Z., Barclay, R.C., and Porter, N.A. (1993) *J. Am. Chem. Soc. 115*, 687–693.
51. Porter, N.A., Kaplan, J.K., and Dussault, P.H. (1990) *J. Am. Chem. Soc. 112*, 1266–1267.
52. Mills, K.A., Caldwell, S.E., Dubay, G.R., and Porter, N.A. (1992) *J. Am Chem. Soc. 114*, 9689–9691
53. Porter, N.A., Mills, K.A., Caldwell, S.E., and Dubay, G.R. (1994) *J. Am. Chem. Soc. 116*, 6697–6705.
54. Chan, H.W.S., Levett, G., and Matthew, J.A. (1979) *Chem. Phys. Lipids 24*, 245–256.
55. Teng, J.I., and Smith, L.L. (1985) *J. Chromatogr. 350*, 445–451.
56. Porter, N.A., and Wujek, D.G. (1984) *J. Am. Chem. Soc. 106*, 2626–2629.
57. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) *J. Am. Chem. Soc. 103*, 6447–6455.
58. Iliou, J.P., Jourdheuil, D., Robin, F., Serkiz, B., Guivarch, P., Lolland, J.P., and Vilaine, J.P. (1992) *Lipids 27*, 959–967.

59. Brash, A.R., Porter, J.D., and Maas, L.B. (1985) *J. Biol. Chem. 260*, 4210–4216.
60. Beckwith, A.L.J., O'Shea, D.M., and Roberts, D.H. (1986) *J. Am. Chem. Soc. 108*, 6408–6409.
61. Porter, N.A., Weber, B.A., Weenen, H., and Khan, J.A. (1980) *J. Am. Chem. Soc. 102*, 5597–5601.
62. Dussault, P.H., and Hayden, M.R. (1992) *Tetrahedron Lett. 33*, 443–446.
63. Bascetta, E., Gunstone, F.D., and Walton, J.C. (1983) *J. Chem. Soc. Perkin Trans. II*, 603–613.
64. Hoffmann, R.W. (1989) *Chem. Rev. 89*, 1841–1860.
65. Haslbeck, F., Grosch, W., and Firl, J. (1983) *Biochim. Biophys. Acta 705*, 185–193.
66. Doba, T., Burton, G.W., and Ingold, K.U. (1983) *J. Am. Chem. Soc. 105*, 6505–6506.
67. Burton, G.W., and Ingold, K.U. (1981) *J. Am. Chem. Soc. 103*, 6472–6477.
68. Niki, E., Kawakami, A., Saito, M., Yamamoto, Y., Tsuchiya, J., and Kamiya, Y. (1985) *J. Biol. Chem. 260*, 2191–2196.
69. Iwatsuki, M., Tsuchiya, J., Komuro, E., Yamamoto, Y., and Niki, E. (1994) *Biochim. Biophys. Acta 1200*, 19–26.
70. Barclay, L.R.C., Baskin, K.A., Dakin, K.A., Locke, S.J., and Vinqvist, M.R. (1990) *Can. J. Chem. 68*, 2258–2269.
71. Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L., and Ingold, K.U. (1985) *J. Am. Chem. Soc. 107*, 7053–7065.
72. Liebler, D.C., Baker, P.F., and Kaysen, K.L. (1990) *J. Am. Chem. Soc. 112*, 6995–7000.
73. Terao, J., and Matsushita, S. (1986) *Lipids 21*, 255–260.
74. Koskas, J.P., Cillard, J., and Cillard, P. (1984) *J. Am. Oil Chem. Soc. 61*, 466–469.
75. Weenen, H., and Porter, N.A. (1982) *J. Am. Chem. Soc. 104*, 5216–5221.
76. Peers, K.E., Coxon, D.T., and Chan, H.W.-S. (1981) *J. Sci. Food Agric. 32*, 898–904.
77. Nagaoka, S., Okauchi, Y., Urano, S., Nagashima, U., and Maukai, K. (1990) *J. Am. Chem. Soc. 112*, 8921–8924.
78. Bruna, E., Petit, E., Beljean-Leymarie, M., Huynh, S., and Nouvelot, A. (1990) *Lipids 24*, 970–975.
79. Khan, J.A., and Porter, N.A. (1982) *Angew. Chem. Int. Ed. 21*, 217–218.
80. Porter, N.A., Wolf, R.A., Yarboro, E.M., and Weenen, H. (1979) *Biochem. Biophys. Res. Commun. 89*, 1058–1064.
81. Porter, N.A., Logan, J., and Kontoyiannidou, V. (1979) *J. Org. Chem. 44*, 3177–3181.
82. O'Connor, D.E., Mihelich, E.D., and Coleman, M.C. (1984) *J. Am. Chem. Soc. 106*, 3577–3584.
83. Porter, N.A., Funk, M.O., Gilmore, D., Isaac, R., and Nixon, J. (1976) *J. Am. Chem. Soc. 98*, 6000–6005.
84. Nugteren, D.H., Vonkeman, H., and Van Dorp, D.A. (1967) *Recueil 86*, 1237–1245.
85. Morrow, J.D., Harris, T.M., and Roberts, L.J. (1990) *Anal. Biochem. 184*, 1–10.
86. Morrow, J.D., Minton, T.A., Mukundan, C.R., Campbell, M.D., Zacker, W.E., Daniel, V.C., Badr, K.R., Blair, I.A., and Roberts, J.L. (1994) *J. Biol. Chem. 269*, 4317–4326.
87. Parthasarathy, S., and Steinberg, D. (1992) *Current Opinion in Lipidology 3*, 313–317.
88. Rosenfeld, M.E., and Lipton, B.A. (1992) *Current Opinion in Lipidology 3*, 318–323.
89. Jialal, I., and Scaccini, C. (1992) *Current Opinion in Lipidology 3*, 324–328.
90. Witzturn, J.L. (1990) in *Hypercholesterolemia, Hypocholesterolemia, Hypertriglyceridemia*, pp. 353–364, Plenum Press, New York.
91. Guyton, J.R., Black, B.L., and Seidel, C.L. (1990) *Am. J. Pathol. 137*, 425–434.
92. Wagner, J.R., Motchnik, P.A., Stocker, R., Sies, H., and Ames, B.N. (1993) *J. Biol. Chem. 278*, 18502–18506.
93. Bowry, V.W., and Stocker, R. (1993) *J. Am. Chem. Soc. 115*, 6029–6044.
94. Ingold, K.U., Bowry, V.W., Stocker, R., and Walling, C. (1993) *Proc. Natl. Acad. Sci. USA 90*, 45–49.
95. Stocker, R., Bowry, V.W., and Frei, B. (1991) *Proc. Natl. Acad. Sci. USA 88*, 1646–1650.
96. Sato, K., Niki, E., and Shimasaki, H. (1990) *Arch. Biochem. and Biophys. 279*, 402–405.
97. Lenz, M.L., Hughes, H., Mitchell, J.R., Via, D.P., Guyton, J.R., Taylor, A.A., Gotto, A.M.J., and Smith, C.V. (1990) *J. Lipid Res. 31*, 1043–1050.

[Received January 17, 1995, and in revised form February 23, 1995; Revision accepted March 8, 1995]

The Gastrointestinal Handling and Metabolism of [1-^{13}C]Palmitic Acid in Healthy Women

Jane L. Murphy[a], Amanda Jones[a], Steven Brookes[b] and Stephen A. Wootton[a,*]

[a]Department of Human Nutrition, University of Southampton, Southampton, SO16 7PX, United Kingdom and [b]Europa Scientific Ltd., Europa House, Electra Way, Crewe, Cheshire, CW1 1ZA, United Kingdom

ABSTRACT: The gastrointestinal handling and metabolism of [1-^{13}C]palmitic acid given as the free fatty acid was examined *in six* healthy women by measuring the excretion of ^{13}C-label in stool and in breath as $^{13}CO_2$. The gastrointestinal handling of [1-^{13}C]palmitic acid was compared with the apparent absorption of dietary lipid by measuring lipid losses in stool. The variation both within and between subjects was determined by repeating the study in the same individuals on separate occasions. The time course for excretion of label in stool over the five-day study period followed a common pattern, with most of the label excreted over the first two days of the stool collection. ^{13}C-Label excreted in stool over the five-day study period was 14.3 ± 9.8% of that administered and on repeating the trial was 31.6 ± 24.7% (not significantly different due to variability); there was poor agreement within subjects. Lipid excreted in stool expressed as a percentage of ingested lipid was 5.2 ± 4.4% in Trial 1 and 5.9 ± 4.0% in Trial 2, and was the same in each individual on repeating the trial. There was no clear relationship between the excretion of ^{13}C-label and lipid in stool (Trial 1: $R = -0.43$, $P > 0.40$; Trial 2: $R = -0.02$, $P > 0.97$). On the first occasion, 22.0 ± 4.5% of the administered label was excreted on breath over the 15-h study period and on repeating the trial was 15.8 ± 9.5% (not significantly different) with poor repeatability in a given individual. There was an inverse relationship between the proportion of ^{13}C-label excreted in stool and that excreted on breath in Trial 1 ($R = -0.80$, $P > 0.06$) with a weaker association observed in Trial 2 ($R = -0.49$, $P > 0.32$). Correcting for differences in the apparent absorption of label reduced the variability in its excretion in breath observed between subjects, particularly in Trial 2. It is concluded that although there are differences in the gastrointestinal handling of [1-^{13}C]palmitic acid both within and between healthy adults, the postprandial oxidation of absorbed substrate was similar. The assumptions underlying these observations need to be examined by characterizing the nature of ^{13}C-label in stool.
Lipids 30, 291–298 (1995).

The factors that determine the gastrointestinal handling of dietary triglycerides (triacylglycerols) and fatty acids and the subsequent metabolic fate in normal, healthy adults are not well understood. Measurements of gross fecal lipid losses made during balance studies in healthy adults (1,2), supported by *in vitro* digestion studies (3,4), have led to the general belief that the apparent absorption of dietary lipid is very efficient, with over 96% of the ingested dietary lipid normally absorbed. Such an approach assumes that all dietary fatty acids are handled equally across the gastrointestinal tract and fails to take into account the contribution made by endogenous losses and bacteria in stool to fecal lipid (5). Balance studies (6,7) and a study using ^{14}C-labelled fatty acids in the rat (8), suggest that both the chain length and degree of unsaturation of fatty acids, as well as the composition and stereospecific structure of triglycerides, may alter the apparent absorption of dietary lipids. Similar observations have been demonstrated in the few balance studies in healthy adults (9,10) and in both children (11) and adults with steatorrhoea (12). Further studies are needed to more precisely define the gastrointestinal handling and metabolic fate of fatty acids in normal health and disease in humans. One approach is to employ orally administered substrates labelled with ^{13}C.

From the rate and time course of excretion of ^{13}C-label in stool and breath $^{13}CO_2$, it is possible to assess the extent to which the labelled substrate is digested, absorbed, and subsequently oxidized by the body. Technical difficulties and the effort associated with the sample preparation and purification of CO_2 prior to analysis have previously limited attempts to measure the excretion of ^{13}C-label in stool. There has been only one published study where the proportion of label excreted in the stool was determined following the ingestion of [1-^{13}C]labelled fatty acids in healthy adults (13). Jones and co-workers (13) demonstrated that different [1-^{13}C]-labelled fatty acids were not handled by the body in the same way. [1-^{13}C]Stearic acid was relatively poorly absorbed by comparison to [1-^{13}C]oleic and linoleic acids, and the proportion of label recovered in the stool varied markedly between subjects (8–35%). However, there was no information on the repeatability of these observations in order to examine the within-subject variation.

We have developed a simplified approach to measure the excretion of ^{13}C in stool using innovative technology in isotope ratio mass spectrometry (14). The aim of the present study was to examine the gastrointestinal handling and me-

*To whom correspondence should be addressed at Department of Human Nutrition, University of Southampton, Bassett Crescent East, Southampton, SO16 7PX United Kingdom.

Abbreviations: PDB, Pee Dee Belemnite limestone; ‰, del per mil (parts per thousand).

Copyright © 1995 by AOCS Press

tabolism of [1-^{13}C]palmitic acid in a group of healthy women by measuring the excretion of ^{13}C-label in stool and on breath as $^{13}CO_2$. We were particularly interested in examining the gastrointestinal handling and metabolism of ^{13}C-labelled palmitic acid administered as the free acid. Palmitic acid is the most predominant long-chain saturated fatty acid in the United Kingdom diet (15) present in the form of mixed triglycerides. We have studied the gastrointestinal handling of [1-^{13}C]palmitic acid administered as the free acid, because it is free palmitic acid that is released from mixed triglycerides following digestion in the small intestine. The gastrointestinal handling of the [1-^{13}C]palmitic acid was compared to the apparent absorption of dietary lipid by measuring lipid in stool, but no relationship was observed. The variation within and between subjects was also examined by repeating the study in the same individuals on a separate occasion.

MATERIALS AND METHODS

Subjects. Six normal, healthy women, aged 21–30 yr, participated in the study. The subjects were members of staff at the University of Southampton. Informed consent was obtained, and the study protocol was approved by the Ethical Committee of Southampton and South West Hampshire Health Commission.

Study protocol. Each subject completed two trials separated by an interval of at least two months. After an overnight fast, the subjects consumed [1-^{13}C]palmitic acid at a dose of 10 mg/kg body weight (99% Atom Percent Excess; Tracer Technologies Inc., Somerville, MA) as part of a controlled standard test meal consisting of 120 g white bread, 100 g orange juice, and 10 g butter (1667 kJ). Butter, which consists predominantly of esterified palmitic acid (16), was used as a vehicle to administer the [1-^{13}C]palmitic acid. Breath samples were collected into breath collection bags (Quintron, Milwaukee, WI) before consuming the test meal to provide a measure of baseline ^{13}C-excretion on breath, and were then collected at hourly intervals for a period of 10 h, and again at 15 h in both trials. Specimen breath samples were transferred into evacuated gas sample containers (Exetainers; Labco, High Wycombe, United Kingdom) for analysis in duplicate. All the subjects were rested for the duration of the tests and were given constant supervision at the Clinical Nutrition and Metabolism Unit at Southampton General Hospital. No additional food or liquids were permitted during the initial 10-h period except for bottled mineral water. After 10 h the subjects consumed a meal without any foods naturally enriched in ^{13}C.

A baseline stool sample was collected on the day before the breath test study period to measure baseline ^{13}C-excretion. Thereafter, all stools passed were collected for five days following administration of the label. Carmine markers were taken with the test meal and at the end of the study to denote the five-day period. Stools were collected separately into polyethylene bags and immediately frozen. The Gompertz and Sammons modification (10) of the method of Van de Kamer *et al.* (17) was employed to measure lipid in stools passed on each day of the study period.

The subjects were instructed to avoid consuming naturally ^{13}C-enriched foods (e.g., corn or maize products, cane sugar) for at least two days prior to and during each five-day study period. None of the animal source protein foods consumed, e.g., eggs, meat, were from animals that consumed a corn-based diet. The same pre-selected diet was consumed throughout both trials to limit any effect that diet may have on the metabolism of [1-^{13}C]palmitic acid. This was achieved by recording the weighed food intake in Trial 1 and repeating the same diet in Trial 2. Within the group, the average contributions made by saturated, polyunsaturated, and monounsaturated fats to dietary energy were 9.3 ± 1.6, 3.4 ± 1.1 and 7.4 ± 1.4%, respectively. The food record from both trials were coded according to the McCance and Widdowson food tables (18) and analyzed using a computerized food composition database (Compeat 4; Lifeline Nutritional Services Ltd., London, United Kingdom).

In addition, the influence of the test meal on ^{13}C-enrichment on breath $^{13}CO_2$ was examined in a third trial on each subject by having the subject consume the same test meal without [1-^{13}C]palmitic acid.

Breath and stool sample analyses. Stools were weighed and homogenized with distilled water, and aliquots were then freeze-dried overnight (Genevac, Ipswich, United Kingdom) to a constant weight. Samples of freeze-dried stool (5 mg) were weighed into tin capsules (Elemental Microanalysis, Okehampton, United Kingdom) for analysis in duplicate. Enrichment of ^{13}C in breath as $^{13}CO_2$ and in stool was analyzed by continuous flow isotope ratio mass spectrometry (ANCA system; Europa Scientific Ltd., Crewe, United Kingdom). The analytical precision for mass spectrometric analyses of reference CO_2 gas and sugar beet calculated from ten samples were (SD) 0.06‰ and 0.03‰, respectively.

Measurement of ^{13}C-enrichment in breath $^{13}CO_2$ and stool. The ^{13}C-enrichment of samples of breath $^{13}CO_2$ and stool was expressed as the per mil relative difference from the reference standard Pee Dee Belemnite limestone (PDB) as defined by Craig ($^{13}C_{PDB}$, ‰) (19). The proportion of administered ^{13}C-label in stool was calculated according to the formula presented by Schoeller and co-workers (20). The enrichment of ^{13}C in each sample is calculated from the increase in the ratio of $^{13}CO_2$ to $^{12}CO_2$ as measured by continuous flow isotope ratio mass spectrometry. This sample ratio is compared with that obtained from a working reference standard, being 5% CO_2 and beet sugar for samples of breath and stool, respectively. The results are then expressed as the "del per mil" (parts per thousand) difference between the $^{13}CO_2/^{12}CO_2$ ratios of the samples and the reference standard. Whole body CO_2 excretion was measured by indirect calorimetry (Deltatrac; Datex Instrumentarium Corporation, Helsinki, Finland) at the same time points as the breath sampling. The proportion of ^{13}C-label excreted in breath $^{13}CO_2$ was expressed as a percent of administered ^{13}C-label calculated from the area under the curve over the 15-h study period.

Measurement of the natural ^{13}C-abundance of the test meal. A test meal was homogenized with 100 g of distilled water, and six aliquots were freeze-dried to a constant weight. Samples from each aliquot of the freeze-dried meal were weighed into tin capsules for analysis in duplicate for enrichment of ^{13}C by isotope ratio mass spectrometry. The natural ^{13}C-abundance of the test meal from duplicate analyses of six separate aliquots of homogenized test meal was −25.5 ± 0.07‰.

Presentation of results and statistical analysis. The results are reported as means ± SD. Statistical comparisons between the data were performed using the paired *t*-test. Differences between means were considered statistically significant where $P < 0.05$. Associations between variables were tested by the Pearson product moment correlation coefficient (R).

RESULTS

Analytical precision. In the present study the average SD for duplicate analyses of breath from all time points was small (less than 0.13‰). Greater variation was observed from duplicate analyses of stool samples collected from all the subjects over the study period (SD 0.85‰). On a separate occasion, the daily variation of ^{13}C-abundance in stools collected over five days was determined within the same individuals consuming no ^{13}C-label or ^{13}C naturally enriched foods. The SD within each individual was no greater than 0.6‰.

Background variation of ^{13}C on breath as $^{13}CO_2$ after an unlabelled test meal. The background variation of ^{13}C on breath was examined in all the subjects by collecting breath samples before consuming an unlabelled test meal, and then at hourly intervals for 10 h, and again at 15 h. Within the group, the pre-meal abundance of ^{13}C on breath was −26.6 ± 0.43‰. There was no detectable increase in the average postprandial excretion of ^{13}C on breath over the 15-h study period (−25.9 ± 0.33, not significantly different from baseline values). The between-subject ^{13}C-variation on breath from all time points was small (average SD 0.41‰). The within subject variation of ^{13}C on breath had an SD of less than 0.63‰.

Excretion of ^{13}C and lipid in stool. Within the group, the baseline abundance of ^{13}C in stool was −26.2 ± 0.75‰ in the first trial. All subjects passed stools on each day of the five-day study period. The greatest levels of enrichment in stool were observed over the first two days of the study period (Fig. 1). The peak enrichment of ^{13}C-label in stool was −15.0 ± 8.3‰. Thereafter the level of ^{13}C-enrichment declined, and after five days the enrichment of ^{13}C in stool was comparable to baseline values. However, there was one exception in the first trial where the enrichment of ^{13}C in stool in subject 3 increased by day five as no label was passed in stool on days two, three, and four of the study period.

On repeating the trial, the baseline abundance of ^{13}C in stool (−25.7 ± 0.50‰), the time course, and peak enrichment (−19.7 ± 73‰) were similar to those in Trial 1. However, much greater variability in the second trial compared to Trial 1 resulted from subject 6, having an exceptionally high level of enrichment of ^{13}C in stool (166.2‰) on the first stool passed.

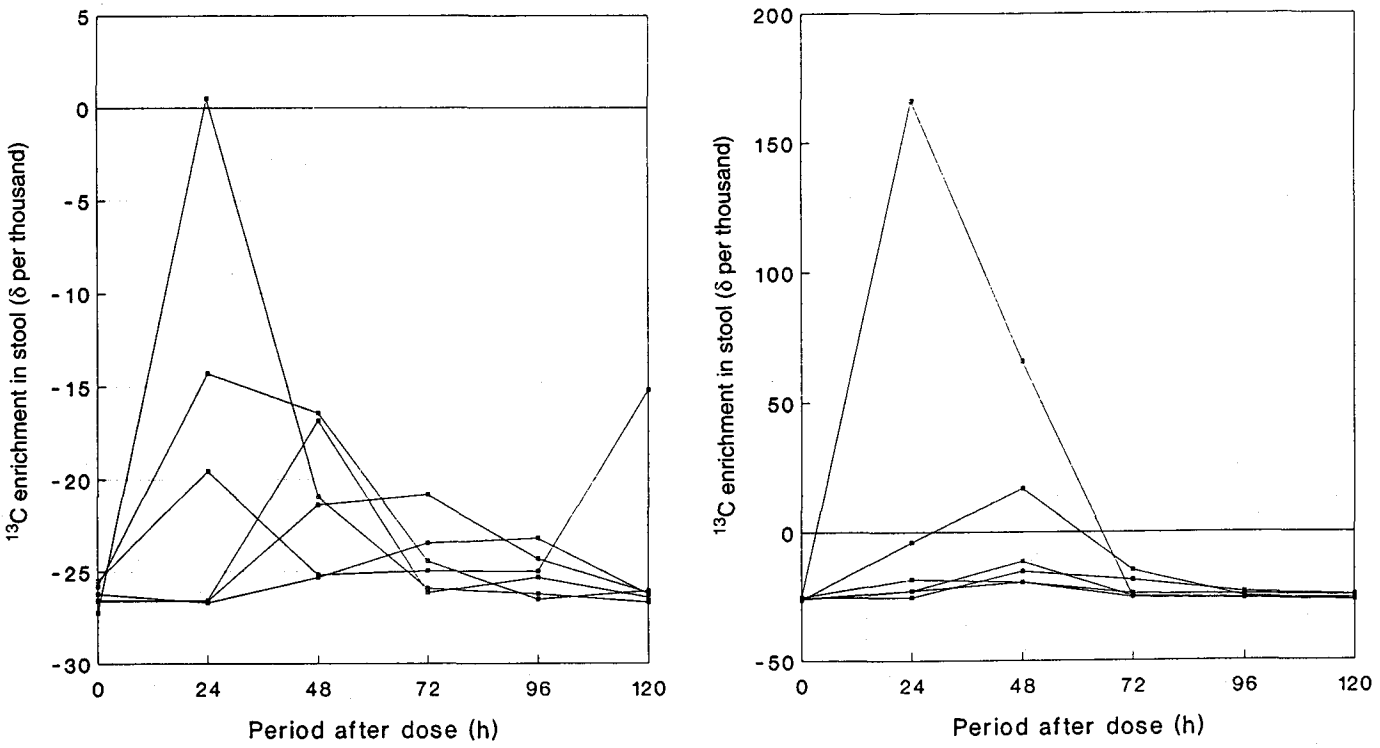


FIG. 1. Time courses for the ^{13}C-enrichment in stool over the five-day study period in six healthy women in Trials 1 and 2. The y-axis is different as one subject in the second Trial had a high enrichment of ^{13}C in the first stool passed.

TABLE 1
The Excretion of Lipid in Stool (% lipid ingested) and the Proportions of ^{13}C-Label Excreted in Stool and on Breath as $^{13}CO_2$ and Retained Within the Body Pools Following Orally Administered [1-^{13}C]Palmitic Acid in Six Healthy Women[a]

Subject number	Stool ^{13}C (% admin[b] ^{13}C)		Stool lipid (% lipid ingested)		Breath $^{13}CO_2$ (% admin ^{13}C)		Body pools (% absorbed ^{13}C)		Breath $^{13}CO_2$ (% absorbed ^{13}C)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
1	7.4	31.5	13.7	13.9	29.0	23.7	69.4	65.6	30.6	34.4
2	13.1	8.3	5.6	5.4	22.3	12.7	74.5	86.1	25.5	13.9
3	17.6	10.0	1.9	3.1	20.1	12.3	75.7	86.3	24.3	13.7
4	7.2	18.9	2.3	4.2	24.2	30.1	74.4	63.3	25.6	36.7
5	32.4	49.9	2.8	4.8	15.7	12.5	77.6	75.2	22.4	24.8
6	8.0	71.0	5.1	3.8	20.4	3.5	77.5	87.8	22.5	12.2
Mean	14.3	31.6	5.2	5.9	22.0	15.8	74.9	77.4	25.2	22.6
SD	9.8	24.7	4.4	4.0	4.5	9.5	3.0	11.0	3.0	11.0

[a]No correction factor has been used to account for the retention of $^{13}CO_2$ in the body pools.
[b]admin, Administered.

In the first trial ^{13}C-label excreted in stool over the five-day study period was 14.3 ± 9.8% of that administered with a fourfold difference between subjects (Table 1). On repeating the trial the ^{13}C-label excreted in stool over the five days was 31.6 ± 24.7% of that administered, with a ninefold difference between subjects. The ^{13}C-label excreted in stool was not significantly different between the trials. There was very poor repeatability in the amount of ^{13}C-label excreted in stool expressed as a proportion of that administered within a given individual.

In the first trial the average dietary intake of lipid over the 5-d study period was 63.6 ± 8.6 g/g, and, on repeating, the trial was 58.9 ± 8.8 g/d (not significantly different). The excretion of lipid in stool was 3.2 ± 2.2 g/d in Trial 1. On repeating the trial, lipid excreted in stool was similar to that observed in the first trial (3.4 ± 2.9 g/d, not significantly different). When expressed as a percentage of that ingested, the excretion of lipid in stool was 5.2 ± 4.4% in Trial 1 and 5.9 ± 4.0% in Trial 2 (not significantly different; Table 1). Within each individual, the excretion of lipid was similar on both occasions even for subject 1, who had a relatively high value for lipid in stool. In both trials there was no relationship between the excretion of ^{13}C-label and lipid in stool (Trial 1: R = –0.43, $P > 0.40$; Trial 2: R = –0.02, $P > 0.97$; Fig. 2).

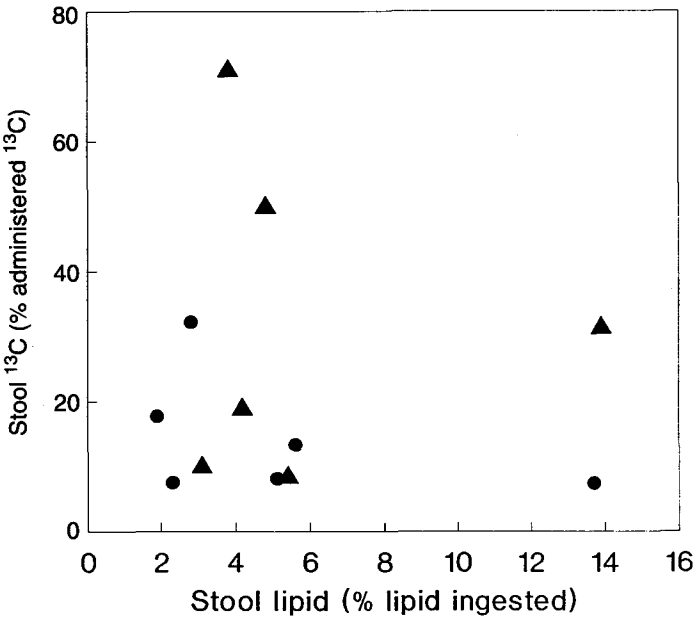


FIG. 2. Stool lipid (% lipid ingested) vs. ^{13}C in stool (% dose administered) in six healthy women in Trials 1 (●) and 2 (▲) (Trial 1: R = –0.43, $P > 0.40$; Trial 2; R = –0.02, $P > 0.97$).

Breath $^{13}CO_2$ excretion measurements. Within the group, the baseline ^{13}C-abundance on breath prior to consuming the test meal was –25.9 ± 0.6‰ in Trial 1. All the subjects exhibited a similar time-course pattern for the excretion of ^{13}C-label on breath over the 15-h study period (Fig. 3). The peak excretion of ^{13}C-label on breath occurred at 6 h (range 4–9 h) after administering the ^{13}C-labelled test meal. The enrichment of ^{13}C on breath at peak excretion was –15.3 ± 2.2‰. At the end of the 15-h study period, the enrichment of ^{13}C on breath had almost returned to the level of baseline ^{13}C-abundance (–22.4 ± 2.0‰). Consuming a test meal 10 h after administering the ^{13}C-label did not increase the excretion of ^{13}C-label on breath as $^{13}CO_2$.

On repeating the trial, the baseline ^{13}C-abundance on breath (–28.0 ± 0.9‰), the time at which peak excretion of ^{13}C-label occurred on breath (6.5 h; 5–8 h), and the time course for excretion of ^{13}C-label on breath over the 15-h study period were similar to Trial 1 (Fig. 3). The enrichment of ^{13}C on breath at peak excretion was less than that observed in Trial 1 (–21.0 ± 2.8‰; $P < 0.01$). The enrichment of ^{13}C on breath at the end of the 15-h study period was closer to baseline ^{13}C-abundance in Trial 2 (–26.0 ± 0.7‰) than that observed in the first trial.

In the first trial, the peak excretion of label on breath when expressed as a proportion of administered ^{13}C was 2.7 ± 0.5%. Over the 15-h study period the excretion of ^{13}C on breath was 22.0 ± 4.5% of that administered (Table 1). On repeating the trial, both the peak excretion of label (1.8 ± 1.0%) and excretion of label on breath over the 15-h study period

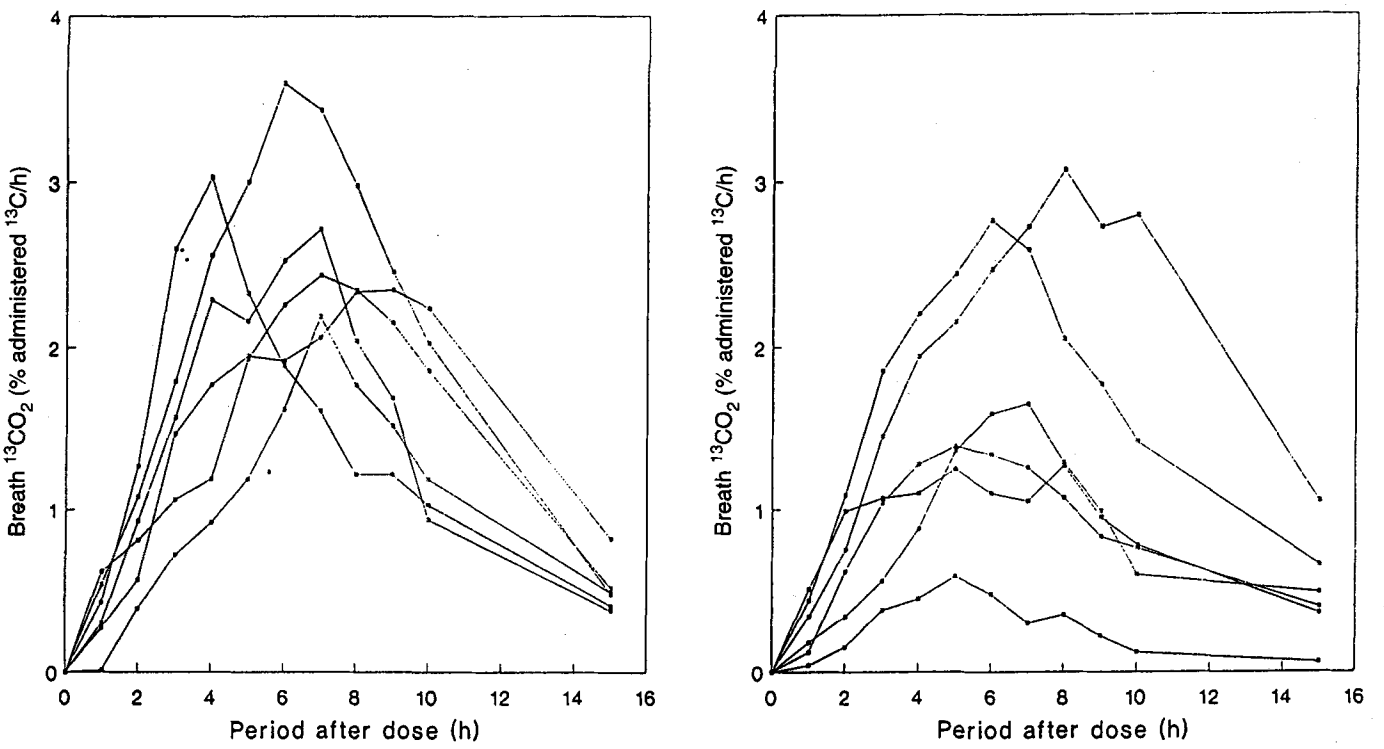


FIG. 3. Time courses for the excretion of ^{13}C on breath as $^{13}CO_2$ over 15 h following oral administration of [1-^{13}C]palmitic acid in six healthy women in Trials 1 (left) and 2 (right).

(15.8 ± 9.5%) tended to be less than that observed in Trial 1 (not significantly different). There was poor repeatability in the amount of ^{13}C-label excreted on breath expressed as a proportion of that administered within a given individual.

There was an inverse relationship between the proportion of ^{13}C-label excreted in stool and that excreted on breath in Trial 1 ($R = -0.80$, $P > 0.06$), with a weaker association observed in Trial 2 ($R = -0.49$, $P > 0.32$; Fig. 4).

Excretion of absorbed ^{13}C on breath as $^{13}CO_2$. The apparent absorption of ^{13}C-label was determined from the difference between label administered and that excreted in stool. The proportion of label excreted on breath $^{13}CO_2$ was then expressed as a proportion of label that was apparently absorbed. Correcting the excretion of ^{13}C-label on breath for differences in apparent absorption reduced the differences between subjects in Trial 1 (2-fold to 1.4-fold difference). Within the group, the amount of ^{13}C-label excreted on breath over the 15-h study period was 25.2 ± 3.0% of that absorbed in Trial 1 (Table 1). On repeating the trial the recovery of absorbed label on breath was 22.6 ± 11.0% (not significantly different to Trial 1). A much greater reduction in the differences between subjects was observed in Trial 2 (ninefold to threefold difference).

^{13}C-label retained within the body pools. The proportion of absorbed label which was not excreted on breath was expressed as retained ^{13}C-label. Within the group, the proportion of absorbed label that was not excreted on breath was 74.9 ± 3.0% in Trial 1 (Table 1). On repeating the trial the proportion of retained ^{13}C-label was similar to Trial 1 (77.4 ± 11.0%). Comparable values were observed between the same subjects on both occasions.

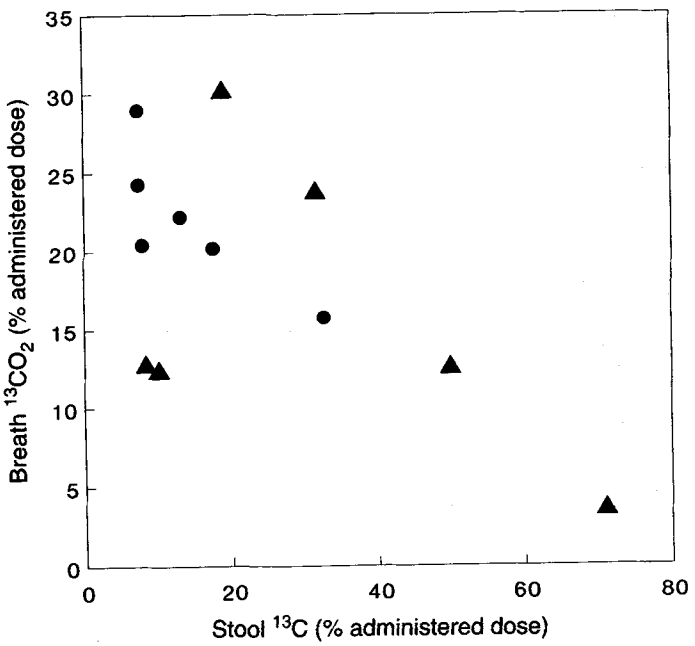


FIG. 4. ^{13}C-Label in stool (% dose administered) vs. ^{13}C-label on breath as $^{13}CO_2$ (% dose administered) in six healthy women in Trials 1 (●) and 2 (▲) (Trial 1: $R = -0.80$, $P > 0.06$; Trial 2: $R = -0.49$, $P > 0.32$).

DISCUSSION

The present investigation was designed to examine the gastrointestinal handling and metabolism of [1-^{13}C]palmitic acid given orally as the free fatty acid in healthy women. From the rate and time course of the excretion of ^{13}C-label in stool and on breath as $^{13}CO_2$, we have determined the extent to which the labelled substrate was apparently absorbed and subsequently oxidized by the body. To our knowledge, this investigation represents the first time that the gastrointestinal handling of [1-^{13}C]palmitic acid has been studied and compared with the handling of dietary lipid in healthy individuals.

Gastrointestinal handling and metabolic fate of [1-^{13}C]palmitic acid. The proportion of ^{13}C-label excreted in stool ranged from 7.4–32.4% within the group and reflected marked differences between healthy subjects in the gastrointestinal handling of [1-^{13}C]palmitic acid. It is not known whether the ^{13}C-label measured in stool is present in the same form as that ingested, i.e., as ^{13}C-labelled palmitic acid. The observed differences between subjects in the gastrointestinal handling of [1-^{13}C]palmitic may be partly accounted for by differences in the nature of label in stool. Further studies are needed to characterize the form of label in stool if the gastrointestinal handling of ^{13}C-labelled substrates are to be interpreted correctly. Assuming that ^{13}C-label excreted in stool is ^{13}C-labelled palmitic acid, the apparent absorption of label would have ranged from 67.6 to 92.6%. The time course for the excretion of label in stool over the five days followed a common pattern, with most of the label excreted in stool over the first two days of the study period.

These results in healthy women are generally similar to those reported for [1-^{13}C]stearic acid by Jones *et al.* (13) in healthy men, and are consistent with those observed from animal studies using ^{14}C-labelled saturated and unsaturated fatty acids (9). Taken together, these observations would suggest that long-chain [1-^{13}C]-labelled saturated fatty acids are not absorbed as well as [1-^{13}C]-labelled unsaturated fatty acids, and that there are wide differences between subjects in the apparent absorption of saturated fatty acids (when ingested as the free fatty acid).

The extent to which the [1-^{13}C]palmitic acid label, administered as the free acid, is handled within the body in the same way as palmitic acid derived by hydrolysis from mixed triglycerides in the diet is not known. At present it is assumed that the [1-^{13}C]-labelled free palmitic acid behaves in the same way as free fatty acids derived from hydrolysis within the lumen of the small intestine. Under normal circumstances, palmitic acid is consumed in the diet in the form of mixed triglycerides predominantly esterified in the *sn*-1 and *sn*-2 positions (16). Hydrolysis of dietary triglycerides by pancreatic lipase and other enzymes, such as carboxyl ester hydrolase in the intestinal lumen, forms 2-monoacylglycerols and fatty acids (2). The products of digestion are absorbed following solubilization with bile salts into mixed micelles. While the 2-monoacylglycerol products of hydrolysis are a well-absorbed form of most fatty acids, it is possible that free fatty acids from the *sn*-1 position may not form mixed micelles as readily as the 2-monoacylglycerol (21). The differences observed in the apparent absorption of [1-^{13}C]palmitic acid between subjects could be attributable to poor solubilization of the fatty acid into micelles. A proportion of the free fatty acids may have formed insoluble soaps with divalent cations in the gastrointestinal tract and may have been excreted in stool (22). Further studies using ^{13}C-labelled mixed triglycerides are required to determine whether the labelled free fatty acid used in the present study is handled in the same manner as a labelled fatty acid esterified into triglycerides at either the *sn*-1 or *sn*-2 positions.

Lipid excreted in stool showed a greater apparent absorption of dietary lipid compared to the gastrointestinal handling of [1-^{13}C]palmitic acid. Differences in the gastrointestinal handling of [1-^{13}C]palmitic acid and lipid were evident from the poor association observed between ^{13}C-label and lipid in stool. This would suggest that [1-^{13}C]palmitic acid was not handled in the same way as lipid in the diet. It may be that the ^{13}C-labelled palmitic acid used in the present study is not a representative form of dietary fatty acids in general, yet is the most frequently occurring fatty acid in terms of its apparent absorption. Alternatively, it could be that the presentation of ^{13}C-labelled palmitic acid to the absorptive site may not be the same as palmitic acid normally hydrolyzed from dietary triglycerides in the gastrointestinal tract. A previous study has shown that emulsified deuterated triglycerides have an apparent absorption above 90% when heated to a temperature of 80°C or above (23), which is similar to that observed in the present study for the apparent absorption of dietary lipid. The [1-^{13}C]palmitic acid label was administered in a form (added to butter) which is different than that for dietary lipid, which is often heated or contains emulsifiers. It could be that part of the discrepancy observed between the apparent absorption of the ^{13}C-labelled palmitic acid and dietary lipid could be attributable to differences in the form of the ingested lipids vs. administered label.

Oxidation of the absorbed ^{13}C-labelled palmitic acid was examined by measuring the excretion of ^{13}C-label on breath as $^{13}CO_2$. It has been shown that the most significant factor which can influence the sensitivity and precision of measurements of ^{13}C on breath as $^{13}CO_2$ is the normal variability of baseline ^{13}C-to-^{12}C ratio (24). In the present study, the between-subject variation in baseline ^{13}C-abundance on breath in Trials 1 and 2, although small, was -25.9 ± 0.6‰ and -28.0 ± 0.9‰, respectively, in healthy women who excluded foods with a high natural ^{13}C-enrichment. The between-subject differences in baseline ^{13}C-abundance values demonstrate the importance of measuring baseline levels in studies examining the excretion of label on breath as $^{13}CO_2$. The proportion of administered ^{13}C-label excreted on breath over the 15-h period ranged from 15.7 to 29.0%. There was a smaller difference between subjects in the proportion of administered ^{13}C-label excreted on breath compared to the variability observed in the gastrointestinal handling of the ^{13}C-labelled palmitic acid. Correcting for differences in the apparent ab-

sorption of label reduced the between-subject variability in oxidation. This would suggest that, once absorbed, the metabolic fate of the label, in particular the oxidation of absorbed substrate, was similar (approximately 20–25%).

On average, the proportion of ^{13}C-label excreted on breath could be underestimated by 13.3% if expressed directly as ^{13}C-label administered rather than as ^{13}C-label made available through absorption. The proportion of ^{13}C-label unaccounted for (not recovered as either stool over 5 d or breath over 15 h) ranged from 69 to 78%. The extent of further oxidation of the label following retention within the body pools after the 15-h study period was not determined. However, limited observations in the same group of individuals demonstrated that the excretion of ^{13}C-label on breath remained at baseline levels after a 24-h study period. Furthermore, not all of the CO_2 generated from the label would have been excreted as breath $^{13}CO_2$. A proportion of the $^{13}CO_2$ formed from oxidation of the labelled substrate is not directly excreted on breath but may be retained in the body pools or excreted *via* urine, skin (25), and stool (26). The body pools are believed to consist of a central, rapidly turning-over, vascular pool which includes CO_2 in the circulation, a peripheral rapidly turning-over pool including organs with a high perfusion rate (heart, brain, liver), and a more slowly turning-over pool which includes muscle, adipose tissue, and bone (27). Previous studies have either presented uncorrected data (28) or have employed correction factors to more accurately predict the extent of oxidation of labelled substrates (29,30). Exactly how much of the CO_2 is retained in the body pools remains unresolved, with estimates ranging from less than 10 to 50% (27). There is a wide range in the reported values for retention of CO_2 in the body pools arising from differences in the type and duration of experiments, and whether the subjects were fasted or fed over the study periods. The values reported in the present study for the oxidation of absorbed label have not been corrected for the retention of $^{13}CO_2$ in the body pools and, as such, are underestimates. Preliminary results of studies from our group have shown that over 24 h, 69% of an intravenous dose of $NaH^{13}CO_3$ could be excreted on breath in the same group of individuals (31). Applying this correction factor to the values in the present study would result in approximately 36.5% of the absorbed label being oxidized as opposed to 25.2%.

These results would suggest that approximately a third of the absorbed [1-^{13}C]palmitic acid was oxidized over the 15-h study period. The values observed are much greater than those reported for the long-chain saturated fatty acid, [1-^{13}C]stearic acid, where only 3% of the absorbed stearic acid was oxidized over the postprandial study period of 9 h (29). It appears that the metabolic fate of [1-^{13}C]stearic acid differs markedly from the observations made for [1-^{13}C]-palmitic acid in the present study despite similarities in the design of the study and in apparent absorption.

Within subject variation in the gastrointestinal handling and metabolic fate of [1-^{13}C]palmitic acid. On repeating the trial to examine within-subject differences, there was a greater range in the gastrointestinal handling of [1-^{13}C]-palmitic acid compared to the first trial. The excretion of ^{13}C-label in stool ranged from 8.3 to 71.0% with poor agreement within each subject. Assuming that ^{13}C-label excreted in stool is ^{13}C-labelled palmitic acid, the apparent absorption of label would have ranged from 29.0 to 91.7%. The greatest difference within a given subject between trials was observed in subject 6, with an apparent absorption of 92% in the first trial and 29% on repeating the trial. This would suggest that, within a given individual single measurement using [1-^{13}C]palmitic acid, label should be interpreted with caution owing to the variability in apparent absorption.

The time course for the excretion of label in stool over the five days followed a similar pattern to that observed in the first trial, with most of the label excreted in stool over the first two days of the study period. In contrast, the lipid excreted in stool was similar to that observed in the first trial, with good agreement within each subject. This would suggest that, on repeating the trial, the apparent absorption of dietary lipid still remained greater than the apparent absorption of [1-^{13}C]-palmitic acid, with no relationship between the two variables.

In the second trial, differences in the proportion of administered ^{13}C that was excreted on breath between subjects were much greater than that observed in the first trial (3.5–23.7%), with poor repeatability within a given individual. There were smaller differences between subjects in the proportion of administered label excreted on breath as compared to the variability in the gastrointestinal handling of labelled substrate, which was similar to that observed in the first trial. Those subjects with the lowest enrichment of ^{13}C on breath tended to exhibit the highest enrichment of ^{13}C in stool.

Although there were greater differences between subjects in the gastrointestinal handling of [1-^{13}C]palmitic acid in the second trial compared to the first trial, the oxidation of absorbed label was similar. On average, the proportion of label excreted on breath could be underestimated by approximately 31% if expressed directly as ^{13}C-label administered rather than as ^{13}C-label made available through absorption. Taken with the observations from the first trial, the effect of correcting for differences in the gastrointestinal handling of label appeared to be small when considered as a group. However, for a given individual, differences in the apparent absorption could markedly alter the interpretation of label excreted on breath. This would suggest that there is a need to account for the amount of ^{13}C-label in stool where a more objective, quantitative approach to examine gastrointestinal handling and metabolism of substrates is required.

In conclusion, the present study has examined the gastrointestinal handling and metabolism of [1-^{13}C]palmitic acid in six healthy women. There were greater differences in the gastrointestinal handling of [1-^{13}C]palmitic acid, both within and between subjects, than the post prandial oxidation of absorbed label. Taken together with the observations by Jones *et al.* (29), it appears that the metabolic fate of [1-^{13}C]-labelled long-chain saturated fatty acids differs markedly despite similarities in apparent absorption. Assuming that the

^{13}C-label excreted in stool is ^{13}C-labelled palmitic acid, the gastrointestinal handling of [1-^{13}C]palmitic acid was different from that observed from dietary lipid in general. The assumption that the ^{13}C-label in stool reflects dietary residue needs to be examined by characterizing the nature of label in stool.

ACKNOWLEDGMENTS

We thank the subjects who participated in the study and Michelle Griffiths for technical assistance. This work was carried out with the support of Scientific Hospital Supplies for J.L.M. and a studentship for A.J.

REFERENCES

1. Wollaeger, E.E., Comfort, M.W., and Osterberg, A.E. (1947) *Gastroenterology 9*, 272–283.
2. Carey, M.C., Small, D.M., and Bliss, C.M. (1983) *Ann. Rev. Physiol. 45*, 651–677.
3. Savary, P., and Desnuelle, P. (1956) *Biochim. Biophys. Acta. 21*, 349–360.
4. Savary, P. (1971) *Biochim. Biophys. Acta. 248*, 149–155.
5. Lusk, G. (1906) in *The Elements of the Science of Nutrition,* 4th edn., Saunders, Philadelphia (reprinted in 1928), pp. 46–60; Academic Press, London (reprinted in 1976).
6. Ockner, R.K., Pittman, J.P., and Yager, J.L. (1972) *Gastroenterology 62*, 981–992.
7. Carroll, K.K. (1958) *J. Nutr. 239*, G141–G150.
8. Kirschner, S.L., and Harris, R.S. (1961) *J. Nutr. 73*, 397–402.
9. Gompertz, S.M., and Sammons, H.G. (1963) *Clin. Chim. Acta. 8*, 591–603.
10. Mitchell, D., McMahon, K.E., Shively, C.A., Apgar, J.L., and Kris-Etherton, P.M. (1989) *Am. J. Clin. Nutr. 50*, 983–986.
11. Fernandes, J., Van de Kamer, J.H., and Weijers, H.A. (1962) *J. Clin. Invest. 21*, 488–494.
12. Pinter, K.G., McCracken, B.H., Lamar, C., Jr., and Goldsmith, G.A. (1964) *Am. J. Clin. Nutr. 15*, 293–298.
13. Jones, P.J.H., Pencharz, P.B., and Clandinin, M.T. (1985) *J. Lab. Clin. Med. 105*, 647– 652.
14. Barrie, A., Davies, J.E., Park, A.J., and Workman, C.T. (1989) *Spectroscopy 4*, 42–52.
15. Gregory, J., Foster, K., Tyler, H., and Wiseman, M. (1990) *The Dietary and Nutritional Status of British Adults,* HMSO, London.
16. Padley, F.B., Gunstone, F.D., and Harwood, J.L. (1986) in *The Lipid Handbook* (Gunstone, F.D., Harwood, J.L., and Padley, F.B., eds.) pp. 49–170, Chapman Hall, London.
17. Van de Kamer, J.H., ten Bokkel Huinink, H., and Weyers, H.A. (1949) *J. Biol. Chem. 177*, 347–355.
18. Paul, A.A., and Southgate, D.A.T. (1985) *McCance and Widdowson's the Composition of Foods,* HMSO, London.
19. Craig, H. (1957) *Geochim. Cosmochim. Acta. 12*, 133–149.
20. Schoeller, D.A., Klein, P.D., and Van Santen, E. (1981) *J. Lab. Clin. Med. 97*, 439–448.
21. Mattson, F.H., and Wolpenhein, R.A. (1964) *J. Biol. Chem. 239*, 2772–2777.
22. Tadayyon, B., and Lutwak, L. (1969) *J. Nutr. 97*, 246–254.
23. Emken, E.E., Adlof, R.O., Rohwedder, W.K., and Gulley, R.M. (1993) *Biochim. Biophys. Acta 1170*, 173–181.
24. Schoeller, D.A., Schneider, J.F., Solomons, N.W., Watkins, J.B., and Klein, P.D. (1977) *J. Lab. Clin. Med. 90*, 412–421.
25. Elia, M., Fuller, N.J., and Murgatroyd, P.R. (1992) *Am. J. Physiol. 263* (*Endocrinol. Metab. 26*), E676–E687.
26. Caprilli, R., Frieri, G., Latella, G., Vernia, P., and Santoro, M.L. (1986) *Digestion 35*, 136–142.
27. Irving, C.S., Wong, W.W., Schuman, R.J., O'Brian Smith, E., and Klein, P.D. (1983) *Am. J. Physiol. 245*, R190–R202.
28. Vantrappen, G.R., Rutgeerts, P.J., Ghoos, Y.F., and Hiele, M.I. (1989) *Gastroenterology 96*, 1126–1134.
29. Jones, P.J.H., Pencharz, P.B., and Clandinin, M.T. (1985) *Am. J. Clin. Nutr. 42*, 769–777.
30. Paust, H., Keles, T., Park, W., and Knoblach, G. (1990) in *Stable Isotopes in Paediatric Nutritional and Metabolic Research* (Chapman, T.E., Berger, R., Reijngoud, D.J., and Okken, A., eds.) pp. 1–22, Intercept, Andover.
31. Jones, A.E., Murphy, J.L., and Wootton, S.A. (1994) *Proc. Nutr. Soc. 53*, 225A.

[Received October 13, 1994; Revision accepted January 27, 1995]

Dietary Fat Alters the Distribution of Cholesterol Between Vesicles and Micelles in Hamster Bile

Bertram I. Cohen*, Takahiro Mikami[1], Nariman Ayyad, Yasuko Mikami[1] and Erwin H. Mosbach

Departments of Surgery, Beth Israel Medical Center, New York, New York and the Albert Einstein College of Medicine of Yeshiva University of the City of New York, The Bronx, New York

ABSTRACT: The type of dietary fat strongly affects the incidence of gallstones in the hamster model of cholesterol cholelithiasis. The present study was designed to determine whether dietary fats could affect gallstone formation by altering the microstructure (vesicular/micellar ratio) of cholesterol in bile. Golden Syrian hamsters from Sasco (Omaha, NE) or Charles River (Wilmington, MA) were fed nutritionally adequate semipurified diets to which were added: (i) 4.0% butterfat without added cholesterol; (ii) 1.2% palmitic acid plus 0.3% cholesterol; or (iii) 4.0% safflower oil plus 0.3% cholesterol. Gallstone incidence and the percentage of cholesterol in vesicles and micelles were determined after two- or six-week feeding periods. Three out of ten Sasco hamsters fed the 1.2% palmitic acid diet for two weeks had cholesterol stones, while none of the eight Charles River animals had stones. In the Sasco hamsters, a significant proportion of the biliary cholesterol was found in void volume vesicles (28.8%) and small vesicles (17.1%); Charles River hamsters had negligible proportions (1.1%) of cholesterol in void volume vesicles and 15.4% in small vesicles. Cholesterol gallstones were most abundant in Sasco hamsters fed 1.2% palmitic acid for six weeks (nine out of ten animals); the mean cholesterol saturation index of the bile was 1.27. A significant proportion of the biliary cholesterol was eluted in the void volume vesicles (21.4%) and in small vesicles (15.0%). Five of the eight identically treated Charles River hamsters had cholesterol stones; the cholesterol saturation index averaged 1.36, and the biliary cholesterol was present in void volume vesicles (31.3%) and small vesicles (14.3%). Vesicles were not detected in the bile of hamsters fed cholesterol-free diets, and none of these animals developed cholesterol gallstones. Safflower oil diets inhibited stone formation even though the cholesterol saturation index was above unity. After six weeks, biliary cholesterol transported in void volume vesicles was highest for Sasco hamsters (13.3%) as compared to Charles River animals (6.9%), but total cholesterol transported in void volume vesicles plus small vesicles was similar in both groups (33.5% vs. 26.2%), respectively. These results suggest that in both strains of hamsters dietary fat influences gallstone formation by modulating the vesicular/micellar distribution of biliary cholesterol. Apparently, the presence of cholesterol/phospholipid vesicles in bile is associated with cholesterol gallstone formation.

Lipids 30, 299–305 (1995).

Animal models of cholesterol cholelithiasis have enabled investigators to study certain aspects of the pathogenesis and course of this important human disease (1–7). In the hamster model, cholesterol cholelithiasis was found to be a function of age, gender, genetic background, composition of the lithogenic diet, and duration of the feeding period (1,2,5).

The animal model developed in our laboratory employed young male golden Syrian hamsters from Sasco, Inc. (Omaha, NE), which were fed nutritionally adequate semipurified diets (SPD) for six weeks. The diets contained 4% butterfat and 0.3% cholesterol. At the end of that period, cholesterol gallstones were present in 50–80% of the animals. Stone incidence was strongly dependent on the type and percentage of fat, in the diet. Thus, substitution of safflower oil for butterfat resulted in a significant decrease in gallstone incidence (6). Butterfat is high in palmitic acid, whereas safflower oil contains more than 75% linoleic acid. Nevertheless, with either fat, the cholesterol saturation index (CSI) of the bile exceeded unity (6). The reason for the observed difference in the incidence of gallstones remained unclear.

In previous studies it was found that human bile was frequently supersaturated with cholesterol but that stones or cholesterol crystals were not necessarily present (8–11). This unexpected result was ascribed to the presence of cholesterol–phospholipid vesicles in bile (12,13). Although such vesicles are inherently unstable and eventually deposit cholesterol crystals, they can persist for hours or days (12,13). It was originally hypothesized that cholesterol was solubilized in bile in the form of cholesterol–phospholipid–bile acid mixed micelles (13). However, studies by Sömjen and Gilat (14,15) and Pattinson *et al.* (16,17) identified large cholesterol–phospholipid vesicles in both hepatic and gallbladder bile. Such metastable vesicles increased cholesterol solubility in bile; they were converted to micelles in the presence of bile

*To whom correspondence should be addressed at Department of Surgery, Beth Israel Medical Center, First Avenue at 16th Street, New York, NY 10003.

[1]Permanent address: Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima 734, Japan.

Abbreviations: CSI, cholesterol saturation index; SPD, semipurified diet.

Copyright © 1995 by AOCS Press

acids. An explanation for the prolonged persistence of vesicles in bile is under intensive study.

We have reported that the presence of cholesterol stones or crystals in gallbladder bile is associated with the presence of relatively large proportions of vesicular cholesterol carriers (18). Other investigators (19) reported differences in cholesterol gallstone incidence from those reported by us (5). These differences were probably due to experimental variables such as animal source, composition of the lithogenic diet, and duration of feeding. The present experiments were undertaken to further investigate the cholesterol carriers in hamster bile in the presence or absence of biliary cholesterol crystals and stones. We hypothesized that differences in cholelithiasis were due to differences in the relative proportions of these cholesterol carriers. Hamsters from two sources [Sasco; and Charles River (Wilmington, MA)] were studied as previous work indicated that stone incidence in these animals differed significantly (5). It was found that the source of the hamsters, the feeding period, and the type of dietary fat affected gallstone incidence and that there was a distinct correlation between the increase of the vesicular/micellar ratio in bile and the development of cholesterol cholelithiasis.

MATERIALS AND METHODS

Animals and diets. Male golden Syrian hamsters (*Mesocricetus auratus*), four weeks of age, weighing between 60 and 70 g, were purchased from either Sasco, Inc. or from Charles River Breeding Laboratories and fed rodent chow for 4–6 d prior to the start of the experiment. Animals from each supplier were randomized into 7–10 animals/group and fed the following for either two or six weeks: (i) SPD plus 4% butterfat (0% cholesterol) (groups 1, 4, 7, 10); (ii) SPD plus 1.2% palmitic acid plus 0.3% cholesterol (groups 2, 5, 8, 11); or (iii) SPD plus 4.0% safflower oil plus 0.3% cholesterol (groups 3, 6, 9, 12). Food intake was monitored throughout the experiment. All animals were fasted 24 h prior to sacrifice to ensure maximum quantities of bile. Animals were anesthetized with 20 mg of ketamine hydrochloride (Aveco Co., Inc., Fort Dodge, IA). Blood was withdrawn *via* cardiac puncture. The gallbladder was visualized, and bile was removed using a Hamilton syringe. The bile was immediately examined under polarized light microscopy for the presence of cholesterol stones and crystals. Bile samples (20–50 μL per animal) from animals in the same group were pooled to obtain adequate quantities (>200 μL) for analysis and centrifuged at 2000 × *g* for 20 min. The supernatant, minimum volume 200 μL, was recovered and equilibrated with 0.25 μCi radiolabeled 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (53 mCi/mmol; Amersham Corp., Arlington Heights, IL) and 0.25 μCi of [1α,2α(n)-^{3}H]cholesterol (45.6 Ci/mmol) (NEN Research Products, Boston, MA) for 60 min at 37°C.

Column chromatography. Cholesterol carriers in bile (vesicles, micelles) were separated on Sepharose CL-4B-200 (Pharmacia, Piscataway, NJ) using the procedure of Pattinson (16,17), with some modifications. Sepharose was equilibrated with buffer (see below) in a 20 × 1.5-cm Pharmacia column. These methods have been described in detail (18). The elution pattern was similar to that described by Pattinson; vesicles were eluted in the void volume (fractions 20–30), followed by small vesicles (fractions 30–45) and micelles (fractions 45–80) (Figs. 1–3). A total of 90 fractions (500 μL each) was collected in 1.5 mL microfuge tubes using an LKB fraction collector. An aliquot from each sample (100 μL) was counted in Aquasol-2 (NEN Research Products) using a Beckman (Palo Alto, CA) 3801 liquid scintillation system. The percentages of cholesterol and phospholipids were determined from the radiolabeled cholesterol and phospholipid present in each sample. All samples were counted for 10 min. Corrections for background and quenching were made using appropriate blanks and standards.

Analytical procedures for biliary lipids. An aliquot of the fresh bile used for column chromatography was analyzed for biliary lipids [determined as described earlier (6)]. The concentration of total lipids in bile was used to calculate the CSI (20).

Statistical analyses. Differences in gallstone incidence between groups were determined using chi-square.

RESULTS

Gallstone incidence at sacrifice is summarized in Table 1. Stone incidence was always higher in Sasco than in Charles River animals. In the absence of exogenous cholesterol, neither Sasco nor Charles River hamsters developed cholelithiasis. Under the conditions employed, dietary palmitic acid (1.2%) gave the highest incidence of gallstones. Sasco hamsters fed palmitic acid had cholesterol gallstones after two weeks (30%, group 2); lithiasis increased significantly to 90% after six weeks (group 5). Charles River hamsters fed the identical diet did not have stones after two weeks (group 8); cholesterol gallstones were detected after six weeks (63%, group 11). Dietary safflower oil significantly reduced the incidence of stones in both Sasco (groups 3 and 6) and Charles River hamsters (groups 9 and 12). Gallstones were present only in Sasco animals fed safflower oil plus 0.3% cholesterol for six weeks (22%, group 6); stones were completely absent from the other safflower oil-fed hamsters (groups 3, 9 and 12).

The biliary lipid composition is also summarized in Table 1. Analyses were performed on the same pooled samples of bile which were used for the chromatographic separation of vesicles and micelles. The CSI of all Sasco hamsters fed cholesterol for either two or six weeks exceeded 1.0 (groups 2, 3, 5, and 6). The bile of Sasco animals fed butterfat diets without cholesterol (groups 1 and 4) was nonlithogenic. In contrast, the bile of Charles River hamsters fed the SPD with either palmitic acid or safflower oil plus cholesterol was lithogenic only after six weeks (groups 11 and 12, CSI = 1.36 and 1.13, respectively). The total lipid concentration (g/dL) tended to be higher in Sasco hamsters compared to Charles River animals fed identical diets (groups 1–6 vs. 7–12, respectively).

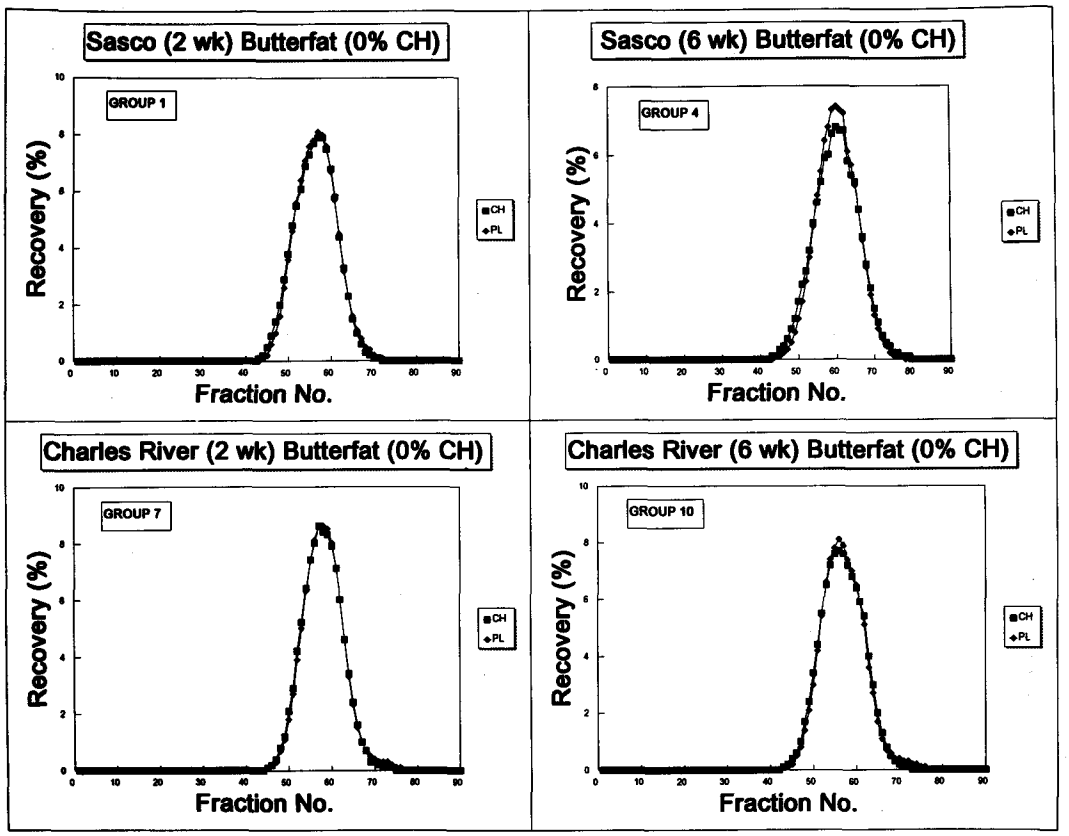


FIG. 1. Gel filtration chromatography of hamster gallbladder bile using Sepharose CL-4B-200 (Pharmacia, Piscataway, NJ). The elution profiles show % recovery of [^{3}H]cholesterol (CH) (■) and [^{14}C]phosphatidylcholine PL (◆). The groups fed 4.0% butterfat + 0% cholesterol are Sasco hamsters (Sasco, Inc., Omaha, NE) (2 wk), group 1; Sasco (6 wk), group 4; Charles River hamsters (Charles River, Wilmington, MA) (2 wk), group 7; Charles River (6 wk), group 10. See experimental section for details of diets, feeding conditions, and chromatography. PL, phospholipids.

The distribution of cholesterol in void volume vesicles, small vesicles, and micelles is summarized in Table 2 and in Figures 1–3. Quantitation was made by determination of ^{3}H and ^{14}C in each fraction. In all experiments in which cholesterol carriers were isolated, the recovery of the radiolabeled cholesterol and phospholipid averaged 90% or better. The cholesterol/phospholipid ratio in the void volume vesicles, small vesicles, and micelles is summarized in Table 2. This ratio was highest for void volume vesicles (range 1.83–2.92), followed by small vesicles (range 0.92–1.90). Small vesicles were detected in the bile of all groups fed 0.3% cholesterol (except Charles River animals fed 4% safflower oil plus 0.3% cholesterol for two weeks, group 9, Table 2). In both Sasco and Charles River animals fed butterfat without cholesterol for either two or six weeks, only micellar cholesterol carriers were present in bile (Table 2 and Fig. 1; groups 1, 4, 7, and 10). The highest amount of cholesterol in void volume vesicles was detected in the bile of Sasco hamsters fed 1.2% palmitic acid plus 0.3% cholesterol for two weeks (28.8%, group 2, Fig. 2). Group 2 was also the only group with cholesterol gallstones at the end of two weeks. However, after six weeks of feeding, both Sasco and Charles River hamsters had a significant proportion of the biliary cholesterol in vesicular form (group 5, 21.4%; group 11, 31.3%). Some Sasco hamsters fed the safflower oil diet for six weeks had cholesterol gallstones (22%). These animals had 13.3% of the biliary cholesterol in void volume vesicles and 20.2% in small vesicles (Table 2, and Fig. 3).

DISCUSSION

The experiments presented here examined whether lithogenic diets produced cholesterol/phospholipid vesicles which nucleated to form cholesterol stones. We separated large vesicles, small vesicles and micelles from gallbladder bile by gel filtration chromatography. The procedure was essentially that of Pattinson (17). Gel filtration was chosen instead of density gradient ultracentrifugation to preserve the metastable vesicles as separations could be carried out in a shorter time (4 vs. 48 h) using smaller quantities (200 μL) of bile. The sizes of the void volume vesicles, small vesicles, and micelles were reported by Pattinson (17) using electron microscopy of the various gel filtration column fractions.

Earlier hamster models of cholesterol cholelithiasis presented problems which made the interpretation of the results difficult. The hamster model developed by Christensen *et al.*

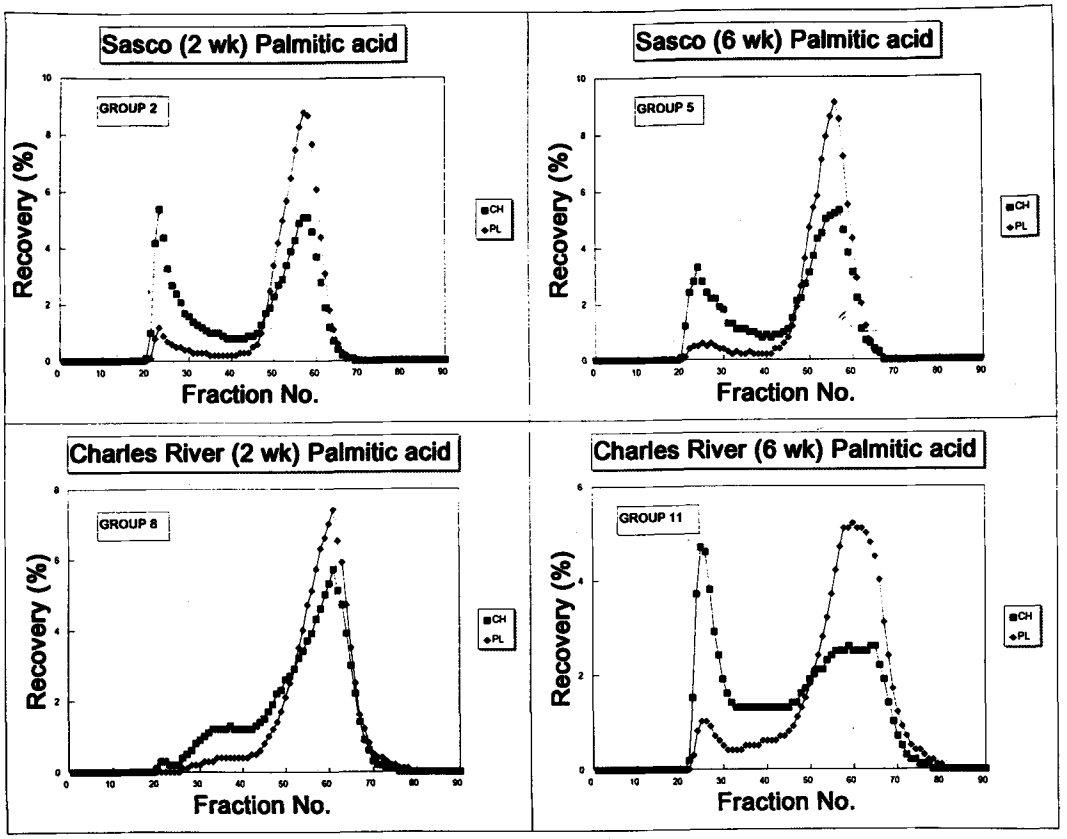


FIG. 2. Gel filtration chromatography of hamster gallbladder bile using Sepharose CL-4B-200. The elution profiles show % recovery of [^{3}H]cholesterol (■) and [^{14}C]phosphatidylcholine (◆). The groups fed 1.2% palmitic acid and 0.3% cholesterol are Sasco (2 wk), group 2; Sasco (6 wk), group 5; Charles River (2 wk), group 8; Charles River (6 wk), group 11. See experimental section for details of diets, feeding conditions, and chromatography. See Figure 1 for abbreviations and source locations.

(2) used diets which lacked essential fatty acids; consequently, even though gallstones formed, many of the hamsters became ill, lost weight, developed diarrhea, and died prematurely. Another hamster model (21) used a chow diet plus cholesterol and estrogen to induce cholesterol gallstones; bile acid therapy prevented stones in these animals. This model could not be duplicated in other laboratories (22). Our laboratory developed a reproducible hamster model of cholesterol cholelithiasis (3–6). In our model, male Sasco hamsters were fed nutritionally adequate SPD supplemented with 0.3% cholesterol to form stones; compounds were added to the diet to study gallstone prevention (7). On the standard lithogenic diet (5), gallstone incidence, after a six-week feeding period, was 50–80%; in all cases, the CSI of the bile exceeded unity. Our studies revealed that gallstone incidence varied significantly with the source of the hamster, e.g., Sasco vs. Charles River (5), even when the CSI in gallbladder bile was above 1.0. We attempted to elucidate dietary factors that affected stone incidence and found that changing the source of fats or fatty acids affected cholelithiasis (23). The CSI of the bile of all animals fed these fats exceeded unity even in the absence of stones.

The "stability" of cholesterol in supersaturated bile which contained neither crystals nor stones, was found to be related to the presence of vesicular rather than micellar cholesterol (14,15). Vesicles, which were rich in cholesterol and phospholipid but contained little bile acids, have now been characterized and identified in human bile (14,15,24–28) and in model bile (29–31). We designed the present study to discover a relationship between certain experimental variables and the distribution of biliary cholesterol between vesicles and micelles. We compared hamsters from two sources (Sasco and Charles River) with different responses to the lithogenic diet. We found that dietary cholesterol was essential for lithogenesis. We also studied the effect of a diet containing safflower oil because with this regimen the bile becomes supersaturated, but few gallstones formed. We used an established procedure to isolate void volume vesicles, small vesicles, and micelles (17), but made some modifications necessary for using hamster bile instead of human bile. These changes were mandated in the hamster model by the small amount of bile (only 20–50 μL/animal) obtainable for analysis. A minimum of 200 μL of bile was needed to obtain adequate separation and quantitation of vesicles and micelles. Statistical analyses were limited because the bile samples were pooled. An additional study of ours using three groups of hamsters (10–12 animals/group) fed 1.2% palmitic acid plus 0.3% cholesterol for six weeks showed similar proportions of void volume vesicles (26.9–34.9%), small vesicles

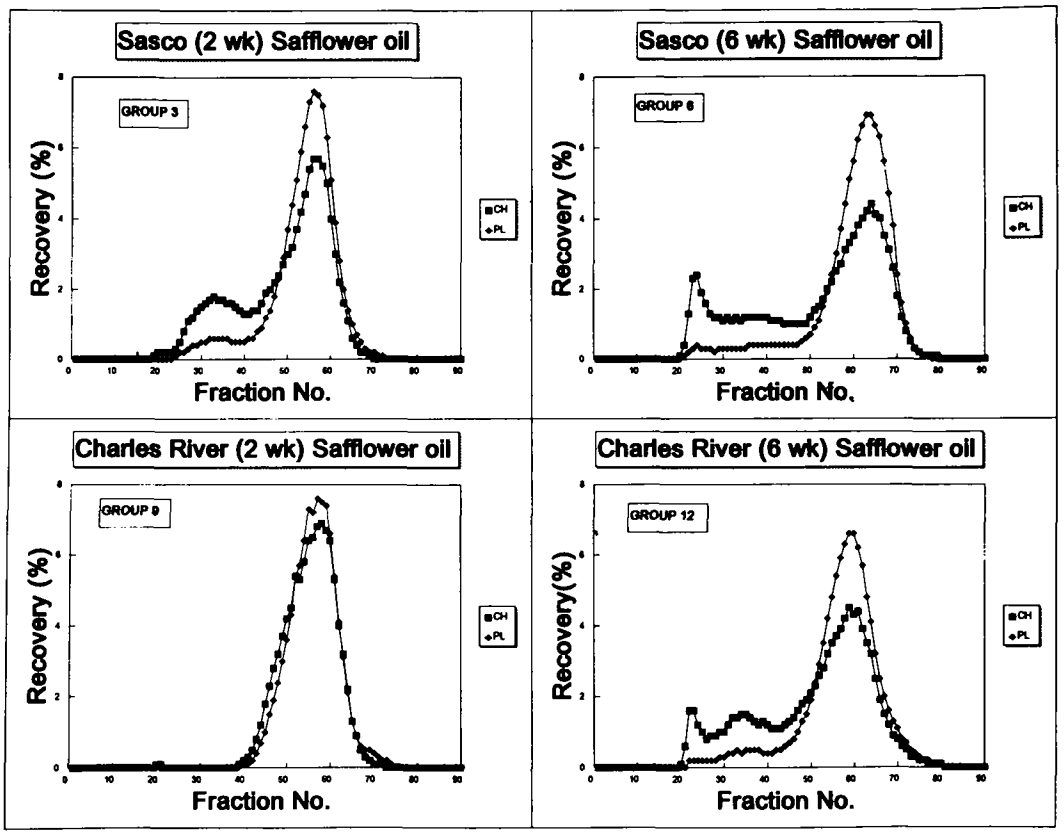


FIG. 3. Gel filtration chromatography of hamster gallbladder bile using Sepharose CL-4B-200. The elution profiles show % recovery of [^{3}H]cholesterol (■) and [^{14}C]phosphatidylcholine (◆). The groups fed 4% safflower oil and 0.3% cholesterol are Sasco (2 wk), group 3; Sasco (6 wk), group 6; Charles River (2 wk), group 9; Charles River (6 wk), group 12. See experimental section for details of diets, feeding conditions, and chromatography. Abbreviations and source locations as in Figure 1.

(12.3–19.0%), and micelles (46.1–59.1%) (18). These results suggest that the analyses of cholesterol carriers are reproducible from experiment to experiment for biles treated under identical experimental conditions. We used the column elution buffer (6 mM sodium taurocholate) described by Pattinson (17). This concentration was at best an estimate. If this amount of bile acid were too low, one might expect to see vesicle formation. No void volume vesicles were detected in four of the twelve groups studied, suggesting that the bile acid concentration used in our eluting buffer was appropriate to maintain the integrity of the void volume vesicles, small vesicles, and micelles. In addition, if the bile acid concentration were too high, one might expect some conversion of vesicles to micelles. In several groups (2, 5, and 11), the total void volume vesicles plus small vesicles exceeded 30%. Our methods allow semi-quantitative comparison between groups; the absolute quantitation of vesicles/micelles may not be possible with current methodology. A similar conclusion was reported by other investigators using human bile (32).

The results presented in Tables 1 and 2 and Figures 1–3 clearly show that void volume vesicles and small vesicles were present in bile from most animals on lithogenic diets but were not present in biles of animals given nonlithogenic diets containing no exogenous cholesterol. The latter groups (1, 4, 7, and 10) carried cholesterol only in the micellar fraction. In the groups fed 1.2% palmitic acid (groups 2, 5, and 11), animals that had cholesterol gallstones retained a significant portion of the biliary cholesterol in void volume vesicles and small vesicles. Charles River hamsters fed the palmitic acid diet for two weeks did not develop gallstones; this was the only group fed palmitic acid which had few (only 1.1%) void volume vesicles in the bile. We postulate that vesicle formation may be the initial event in formation of cholesterol crystals and cholesterol stones in this model. The presence of vesicular cholesterol in the bile of gallstone patients has been reported (17). The ratio of cholesterol/phospholipid in human bile was highest in void volume vesicles, followed by small vesicles and micelles (16,17). A similar trend was observed for these particles in our hamsters. It has been suggested that a high cholesterol/phospholipid ratio would accelerate nucleation with subsequent crystal formation (25). Interestingly, we found the highest cholesterol/phospholipid ratio in animals receiving safflower oil plus cholesterol (group 6, 2.83; group 12, 2.92). We found that the cholesterol/phospholipid ratio did not correlate with changes in total lipids; a similar finding was reported for human bile (16,17). For example, in Sasco hamsters, group 2, fed SPD + 1.2% palmitic acid + 0.3% cholesterol, total lipids were higher compared to group

TABLE 1
Gallstones and Biliary Lipids at Sacrifice[a]

Group number	Source	Feeding period	Diet	Cholesterol		Bile acids (mM)	Phospholipids (mM)	Cholesterol (mM)	Total lipids (g/dL)	Cholesterol saturation index
				Stones	Crystals					
1	Sasco	2 wk	SPD + 4% butterfat + 0% cholesterol	0/8	0/8	220.2	22.1	4.1	12.7	0.41
2	Sasco	2 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	3/10	3/10	223.8	15.7	8.8	12.6	1.03
3	Sasco	2 wk	SPD + 4% safflower oil + 0.3% cholesterol	0/8	0/8	132.5	32.4	11.9	9.5	1.07
4	Sasco	6 wk	SPD + 4% butterfat + 0% cholesterol	0/9	0/9	206.0	15.6	5.3	11.5	0.67
5	Sasco	6 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	9/10[b]	9/10[b]	135.3	27.8	12.7	9.3	1.27
6	Sasco	6 wk	SPD + 4% safflower oil + 0.3% cholesterol	2/9	2/9	170.5	21.8	10.2	10.5	1.27
7	Charles River	2 wk	SPD + 4% butterfat + 0% cholesterol	0/8	0/8	153.8	26.4	2.5	9.7	0.27
8	Charles River	2 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	0/8	0/8	125.1	25.3	7.7	8.4	0.90
9	Charles River	2 wk	SPD + 4% safflower oil + 0.3% cholesterol	0/8	0/8	129.8	24.5	7.0	8.5	0.81
10	Charles River	6 wk	SPD + 4% butterfat + 0% cholesterol	0/7	0/7	88.5	9.4	2.3	5.2	0.65
11	Charles River	6 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	5/8[c]	6/8[c]	79.5	19.1	8.2	5.7	1.36
12	Charles River	6 wk	SPD + 4% safflower oil + 0.3% cholesterol	0/8	0/8	109.9	24.2	9.2	7.6	1.13

[a]Animals were fed the semipurified diets (SPD) for either two or six weeks. See Experimental section for details. Sources: Sasco, Omaha, NE; Charles River, Wilmington, MA. [b]Differs significantly from groups 1, 3, 4, and 6, $P < 0.01$ by chi-square. [c]Differs significantly from groups 7–9, 10, and 12, $P < 0.01$ by chi-square.

6, fed SPD + 4% safflower oil + 0.3% cholesterol (12.6 vs. 10.5 g/dL, respectively), yet the cholesterol/phospholipid ratio in vesicles was higher in group 6 vs. group 2 (2.83 vs. 2.61, respectively). We reported earlier (18) that vesicles were present in hamster bile in animals fed 4% butterfat + 0.3% cholesterol for six weeks. However, only micelles were found when either 1.2% palmitic acid or 4% safflower oil were fed without exogenous cholesterol (Cohen, B.I., and Mosbach, E.H., unpublished data).

Cholesterol gallstone formation is affected by factors other than percent vesicles and micelles in bile. Recent studies showed that specific proteins in bile were associated with the vesicular fraction and presumably affected the nucleation of cholesterol (33–37). However, the limited quantities of hamster bile available have precluded such studies to date. Biliary phospholipids have also been implicated in the stability of cholesterol vesicles in bile (37,38). It was suggested that dietary changes in the fatty acid composition of biliary phosphatidycholines could affect vesicle stability (37–39). To date, we have not characterized individual phosphatidylcholine species under the conditions studied in the present experiments.

In summary, we report the composition of the cholesterol carriers in bile obtained from Sasco and Charles River hamsters fed nutritionally adequate lithogenic or nonlithogenic diets for two or six weeks. The presence of cholesterol gallstones and cholesterol crystals was correlated in both strains of hamsters with the proportions of void volume vesicles and small vesicles in bile.

ACKNOWLEDGMENTS

Supported in part by USPHS grants R01 DK-43204 from the National Institute of Diabetes and Digestive and Kidney Disease (BIC), R37 HL-24061 from the National Heart, Lung, and Blood Institute (EHM), and a grant from the Singer-Hellman Fund.

REFERENCES

1. Brenneman, D.E., Connor, W.E., Forker, E.L., DenBesten, L. (1972) *J. Clin. Invest. 51*, 1495–1503.
2. Christensen, F., Dam, H., and Kristensen, G. (1956) *Acta Physiol. Scand. 36*, 329–336.
3. Cohen, B.I., Mosbach, E.H., McSherry, C.K., Stenger, R.J., Kuroki, S., and Rzigalinski, B. (1986) *Hepatology 6*, 874–880.
4. Cohen, B.I., Setoguchi, T., Mosbach, E.H., McSherry, C.K., Stenger, R.J., Kuroki, S., and Soloway, R.D. (1987) *Am. J. Surg. 153*, 130–138.
5. Cohen, B.I., Matoba, N., Mosbach, E.H., and McSherry, C.K. (1989) *Lipids 24*, 151–156.
6. Cohen, B.I., Mosbach, E.H., Ayyad, N., Miki, S., and McSherry, C.K. (1992) *Lipids 27*, 526–532.
7. Cohen, B.I., Matoba, N., Mosbach, E.H., Ayyad, N., Hakam, K., Suh, S.O., and McSherry, C.K. (1990) *Gastroenterology 98*, 397–405.
8. Holzbach, R.T. (1983) in *Gastrointestinal Disease* (Schlesinger, M.H., and Fordtran, J.S., eds.), pp. 1356–1373, W.B. Saunders Co., Philadelphia.

TABLE 2
Distribution of Cholesterol Among Its Carriers in Hamster Bile[a]

Group number	Source	Feeding period	Diet	Cholesterol%			Cholesterol/Phospholipid		
				Vesicles[b]	Small vesicles	Micelles	Vesicles[a]	Small vesicles	Micelles
1	Sasco	2 wk	Semipurified diet (SPD) + 4% butterfat + 0% cholesterol	—	—	100.0	—	—	0.19
2	Sasco	2 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	28.8	17.1	54.1	2.61	1.60	0.34
3	Sasco	2 wk	SPD + 4% safflower oil + 0.3% cholesterol	0.6	22.9	76.5	—	1.11	0.32
4	Sasco	6 wk	SPD + 4% butterfat + 0% cholesterol	—	—	100.0	—	—	0.34
5	Sasco	6 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	21.4	15.0	63.6	2.44	1.90	0.32
6	Sasco	6 wk	SPD + 4% safflower oil + 0.3% cholesterol	13.3	20.2	66.5	2.83	1.48	0.34
7	Charles River	2 wk	SPD + 4% butterfat + 0% cholesterol	—	—	100.0	—	—	0.09
8	Charles River	2 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	1.1	15.4	83.5	—	0.93	0.27
9	Charles River	2 wk	SPD + 4% safflower oil + 0.3% cholesterol	0.3	—	99.7	2.04	—	0.29
10	Charles River	6 wk	SPD + 4% butterfat + 0% cholesterol	—	—	100.0	—	—	0.24
11	Charles River	6 wk	SPD + 1.2% palmitic acid + 0.3% cholesterol	31.3	14.3	54.4	1.83	0.92	0.27
12	Charles River	6 wk	SPD + 4% safflower oil + 0.3% cholesterol	6.9	19.3	73.8	2.92	1.17	0.30

[a]See Table 1 for company sources. [b]Void volume vesicles.

9. Admirand, W.H., and Small, D.M. (1968) *J. Clin. Invest. 47,* 1043–1052.
10. Carey, M.C., and Small, D.M. (1978) *J. Clin. Invest. 61,* 998–1026.
11. Vlahcevic, Z.R., Bell, C.C., Jr., and Swell, L. (1970) *Gastroenterology 59,* 62–69.
12. Carey, M.C., and Lamont, J.T. (1992) *Prog. Liver Dis. 10,* 139–163.
13. Lamont, J.T., and Carey, M.C. (1992) *Prog. Liver Dis. 10,* 165–191.
14. Sömjen, G.J., and Gilat, T.A. (1983) *FEBS Lett. 156,* 265–268.
15. Sömjen, G.J., and Gilat, T.A. (1985) *J. Lipid Res. 26,* 699–704.
16. Pattinson, N.R. (1985) *FEBS Lett. 181,* 339–342.
17. Pattinson, N.R., Willis, K.E., and Frampton, C.M. (1991) *J. Lipid Res. 32,* 205–214.
18. Mikami, T., Cohen, B.I., Mikami, Y., Ayyad, N., and Mosbach, E.H. (1994) *Lipids 29,* 529–534.
19. Trautwein, E.A., Liang, J., and Hayes, K.C. (1993) *Comp. Biochem. Physiol. 104A,* 829–835.
20. Kuroki, S., Cohen, B.I., Carey, M.C., and Mosbach, E.H. (1986) *J. Lipid Res. 27,* 442–446.
21. Pearlman, B.J., Bonorris, G.G., Phillips, M.J., Chung, A., Vimadalal, S., Marks, J.W., and Schoenfield, L.J. (1979) *Gastroenterology 77,* 634–641.
22. Mosbach, E.H., Singhal, A.K., and McSherry, C.K. (1983) in *Bile Acids and Cholesterol in Health and Disease* (Paumgartner, G., Stiehl, A., and Gerok, W., eds.), pp. 151–155, MTP Press, Lancaster, England.
23. Ayyad, N., Cohen, B.I., Mosbach, E.H., and Miki, S. (1992) *Lipids 27,* 993–998.
24. Chijiiwa, K., Hirota, I., and Noshiro, H. (1993) *Dig. Dis. Sci. 38,* 161–166.
25. Halpern, Z., Dudley, M.A., Kibe, A., Lynn, M.P., Breuer, A.C., and Holzbach, R.T. (1986) *Gastroenterology 90,* 875–885.
26. Lee, S.P., Park, H.Z., Madani, H., and Kaler, E.W. (1987) *Am. J. Physiol. 252,* G374–G383.
27. Harvey, P.R.C., Sömjen, G., Gilat, T., Gallinger, S., and Strasberg, S.M. (1988) *Biochim. Biophys. Acta 958,* 10–18.
28. Whiting, M.J., and Watts, J.M. (1984) *Gastroenterology 86,* 243–248.
29. Donovan, J.M., and Carey, M.C. (1990) *Hepatology 12,* 94S–105S.
30. Donovan, J.M., Timofeyeva, N., and Carey, M.C. (1991) *J. Lipid Res. 32,* 1501–1512.
31. Donovan, J.M., and Jackson, A.A. (1993) *J. Lipid Res. 34,* 1121–1129.
32. Hussaini, H.S., Murphy, G.M., Kennedy, C., Besser, M.G., Wass, J., and Dowling, R.H. (1994) *Gastroenterology 107,* 1503–1513.
33. Rigotti, A., Nuñez, L., Amigo, L., Puglielli, L., Garrido, J., Santos, M., Gonzalez, S., Mingrone, G., Greco, A., and Nervi, F. (1993) *J. Lipid Res. 34,* 1883–1894.
34. LaRusso, N.F. (1984) *Am. J. Physiol. 247,* G199–G205.
35. Reuben, A. (1984) *Hepatology 4,* 46S–50S.
36. Miquel, J.F., Rigotti, A., Rojas, E., Brandan, E., and Nervi, F. (1992) *Clin. Sci. 82,* 175–180.
37. Booker, M.L., LaMorte, W.W., Ahrendt, S.A., Lillemoe, K.D., and Pitt, H.A. (1992) *J. Lipid Res. 33,* 1485–1492.
38. Gilat, T., Halpern, Z., Peled, Y., and Sömjen, G. (1990) *Gastroenterol. Int. 3,* 81–86.
39. Holan, K.R., Holzbach, R.T., Hsieh, J., Welch, D.K., and Turcotte, J.G. (1979) *Digestion 19,* 251–258.

[Received July 18, 1994; Revision accepted January 17, 1995]

Cholesteryl Ester Transfer Protein Inhibition in Hypercholesterolemic Hamsters: Kinetics of Apoprotein Changes

Steven H. Zuckerman* and Glenn F. Evans

Division of Cardiovascular Research, Lilly Research Labs, Indianapolis, Indiana 46285

ABSTRACT: Inhibition of cholesteryl ester transfer protein (CETP) activity in hypercholesterolemic hamsters results in elevated high-density lipoprotein (HDL) cholesterol, an increase in HDL size, and the appearance of apolipoprotein E (apo E)-rich, apo A-I-poor particles. The present study has focused on the kinetics of apoprotein redistribution among the HDL particles and the relative increase in HDL-associated apo E and CETP in hypercholesterolemic hamsters, following inhibition of transfer activity using the monoclonal antibody, TP2. A 60% inhibition in CETP activity was observed 24 h after antibody injection and was associated with an increase in HDL cholesterol and HDL size. Increased amounts of apo E were associated with these HDL particles and remained in this fraction throughout the duration of the study. In contrast, while CETP was also detected on large HDL particles, this distribution shifted back toward the pretreatment pattern by 14 d. The dynamic changes in apoprotein distribution may represent a compensatory physiologic response following disruption of reverse cholesterol transport.
Lipids 30, 307–311 (1995).

Cholesteryl ester transfer protein (CETP), a 74 kD hydrophobic glycoprotein, mediates the transfer of cholesterol ester from high-density lipoprotein (HDL) to acceptor apoprotein B-containing lipoproteins and a reciprocal transfer of triglycerides to HDL (1,2). The role of CETP in the process of reverse cholesterol transport has been suggested through studies of CETP-deficient families (3,4), in rabbits or hamsters in which CETP activity has been inhibited (5–7), and in murine CETP transgenic models (8,9). CETP deficiency in humans is associated with the accumulation of large apoprotein E (apo E) containing HDL particles, HDL_c, increased amounts of HDL cholesterol, decreased low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol, and increased heterogeneity in LDL particle size (3,4,10–12). In addition to the appearance of apo A-I-poor, apo E-rich large HDL, the ratio of HDL_2 to HDL_3 particles was increased in subjects homozygous for the splicing defect in intron 14 of the CETP gene (3,11).

While CETP deficiency is associated with increased HDL and a less atherogenic profile, the consequences of increased CETP activity have been investigated in transgenic mice. C57BL/6 mice expressing either the human or simian CETP gene demonstrate a decrease in HDL cholesterol following transgene induction (8,9) and develop arterial lipid lesions when maintained on a cholesterol-rich diet (9). Increased CETP has also been observed clinically in some patients presenting with dyslipidemia (13,14), nephrotic syndrome (15), and following probucol therapy (16). Serum cholesterol and lipoprotein profiles reflect the increase in CETP activity by an overall decrease in HDL cholesterol and a shift in the ratio of HDL_2 to HDL_3, with an increase in the amount of the smaller HDL_{3b} particles (17).

The inverse relationship between CETP and HDL levels in both human and in animal models suggests that pharmacologic inhibition of CETP synthesis or activity may mitigate the progression of atherosclerosis. This approach, however, requires verification in small animal models that are amenable to drug discovery. Syrian golden hamsters, in contrast to mice and rats, have a significant portion of serum cholesterol associated with the LDL fraction (18–20). Hypercholesterolemia in this model results in an increase in LDL and VLDL cholesterol and a 2–3-fold increase in plasma CETP activity (18–22). Inhibition of CETP activity in normocholesterolemic and hypercholesterolemic hamsters by subcutaneous injection of the CETP-neutralizing monoclonal antibody TP2 (23) results in increased HDL cholesterol, and in hypercholesterolemic animals, the association of apo E and CETP with larger HDL species (7). However, whether these apoprotein changes occur early following inhibition of CETP activity and what the duration is of these effects relative to the return of serum transfer activity were questions which required further investigation.

In the present study, these two questions which related to the kinetics of apoprotein redistribution onto larger HDL particles, have been investigated. In addition to this shift in apo E and CETP, the inhibition of transfer activity results in a significant increase in the amount of apo E and CETP on the HDL particle. Apoprotein changes were observed 24 h after

*To whom correspondence should be addressed.

Abbreviations: apo E, Apoprotein E; CETP, cholesteryl ester transfer protein; ECL, enhanced chemiluminescence; FPLC, fast-performance liquid chromatography; HDL, high-density lipoproteins; IOD, integrated optical density; LDL, low-density lipoproteins; VLDL, very low density lipoproteins.

Copyright © 1995 by AOCS Press

the initial TP2 injection and were still apparent at 14 d, a point at which the cholesterol profile resembled the immunoglobulin control. Therefore, accumulation of apo E-rich, apo A-I-poor HDL in CETP-deficient hypercholesterolemic hamsters represents an early response to inhibition of transfer activity, and these particles persist at a time point at which transfer activity and serum cholesterol profiles approach the pattern seen in control hamsters.

METHODS

Hamster model and CETP assay. Male Syrian hamsters between 120–175 g (Charles River Laboratories, Portage, MI) were maintained on a cholesterol-rich diet (Purina 5001 rodent chow, supplemented with 10% saturated coconut oil and 0.12% cholesterol) for a minimum of one week prior to use. Hypercholesterolemic hamsters, with serum cholesterols greater than 200 mg/dl, were injected subcutaneously at days 0 and 4 with either 12.5 mg/kg of TP2 monoclonal antibody or a control IgG (Cappel, West Chester, PA). An ascites preparation of TP2 antibody (23) was obtained by injection of pristane primed Balb/c mice with the TP2 hybridoma cell line (provided by Dr. Allan Tall, Columbia University, NY). At designated intervals, serum was obtained by retroorbital bleeding. Preparation of [^{3}H]-cholesterol ester-labeled HDL and CETP transfer assays were performed using minor modifications (7) of previously described procedures (13,24).

Serum lipoprotein and electrophoretic analysis. Changes in HDL, LDL, and VLDL were evaluated by fast-performance liquid chromatography (FPLC) (Pharmacia, Bromma, Sweden) analysis using tandem Superose 6 columns (Pharmacia) (25) with cholesterol determined on the fractions as described previously (7). FPLC fractions 20–40, including the LDL and HDL peaks, were electrophoresed on denaturing 10–20% gradient gels, and electroblotted onto nitrocellulose using semiwet blotting conditions (ABN Polyblot; American Bionetics, Inc. Emeryville, CA). Nitrocellulose blots were probed with polyclonal goat antisera against human apo E (Chemicon, Temecula, CA) or with TP2, and immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Amersham Intl., Buckinghamshire, England) as previously described (7). Film (Hyperfilm-ECL; Amersham) was exposed to the chemiluminescent signal under conditions which resulted in a linear film exposure. Bands were quantitated by densitometry and expressed as integrated optical density (IOD) units using a Truvel scanner and Visage software (Bio Image Products, Ann Arbor, MI).

RESULTS

The injection of 12.5 mg/kg of TP2, a concentration of antibody that results in maximal inhibition of CETP activity in hypercholesterolemic hamsters (7), induced a significant shift in the lipoprotein profile when compared to the IgG control. An increase in both HDL size and cholesterol content was apparent 24 h after injection, was maximal between days 4–9, and was less significant by day 11 (Fig. 1). Concomitant with

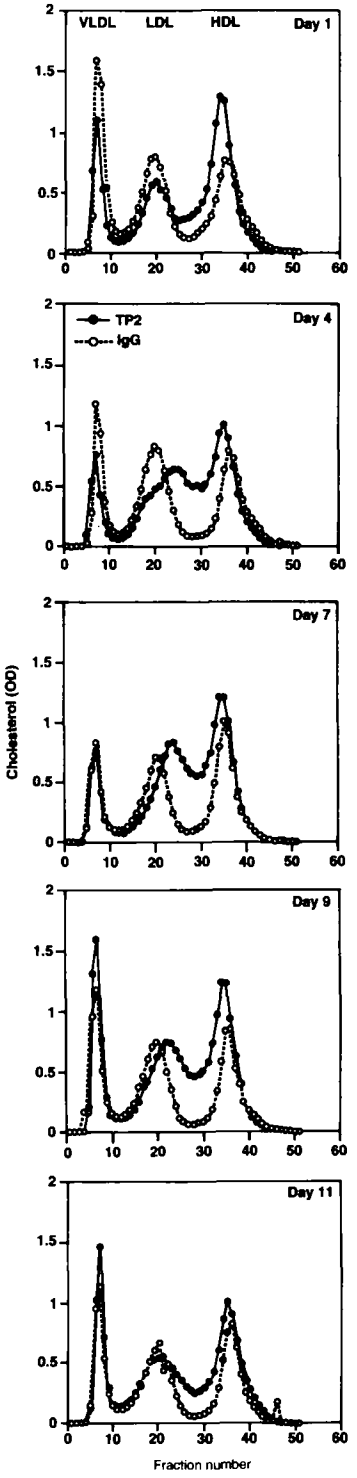


FIG. 1. Effect of cholesteryl ester transfer protein (CETP) inhibition on serum lipoprotein cholesterol profiles. Hamsters injected with TP2 or an IgG (Cappel, West Chester, PA) control were bled at days 1, 4, 7, 9, and 11; 250 mL sera pools (six animals per group) were subjected to fast-performance liquid chromatography on tandem linked Superose 6 columns (Pharmacia, Bromma, Sweden). Resulting fractions were monitored for total cholesterol; very low density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) peaks were identified using purified lipoprotein standards. CETP activity was inhibited: 60%, day 9; 30%, day 11. Reduction in transfer activity relative to day 0 was significant by a two-tailed Student's *t*-test for all points evaluated ($P < 0.001$); OD, optical density.

the increase in HDL cholesterol, there was a reduction in VLDL and LDL cholesterol which was also detected at 24 h. While the reduction in VLDL and LDL cholesterol during the earlier time points was reproducible, the increase in VLDL cholesterol 9 and 11 d after TP2 injection was more variable.

In addition to the increase in HDL cholesterol, there was a significant shift in the distribution and amount of apo E on the lipoproteins. Apo E in the hypercholesterolemic hamster was primarily localized to the ascending shoulder of the HDL peak on FPLC, with the greatest concentration of apo E occurring between fractions 30–32 (Fig. 2A). Inhibition of CETP activity resulted in both a significant shift in apo E distribution to larger lipoprotein particles between fractions 22–30 and in the amount detected. These qualitative and quantitative effects on apo E were apparent by 24 h, the earliest time point evaluated (Table 1). The shift in apo E to larger particles was still apparent at days 11 and 14. While some shift in apo E relative to day 0 was also apparent in IgG-treated hamsters (Fig. 2B), these effects were less marked with most of the apo E detected between fractions 28–30.

TABLE 1
Quantitative Changes in apo E and CETP in TP2-Treated Hamsters

Days[a]	Apo E (IOD)[b]	CETP (IOD)[c]
0	0.15	0.227
1	0.61	12.80
4	5.61	11.47

[a]Days post-injection of 12.5 mg/kg of TP2.
[b]Integrated optical density (IOD), units were based on the peak fraction containing the greatest amount of apoprotein E (apo E). This represented fraction 30 from the day 0 pool and 26 at both the day 1 and day 4 pools.
[c]IOD units were based on the peak cholesteryl ester transfer protein (CETP)-containing fractions, which was fraction, 30 for the day 0 pool and 28 at both the day 1 and day 4 pools. Sixteen-second and fifteen-minute exposures were scanned for apo E and CETP, respectively. These exposures were determined to be within the linear adsorption range.

The profile for CETP was then evaluated to determine whether the apoprotein changes observed following CETP inhibition were unique to apo E (Fig. 3). The untreated day 0 hypercholesterolemic hamsters had maximal CETP detected by immunoblot between fractions 30–34. Twenty-four hours after the initial TP2 injection, there was both an increase in

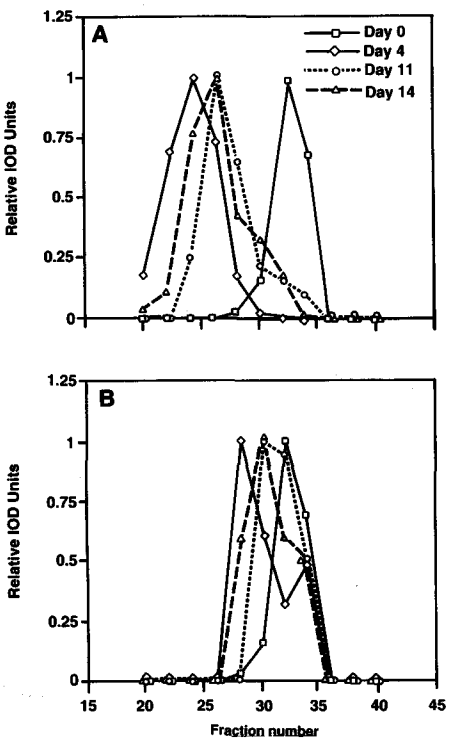


FIG. 2. Kinetics of apoprotein E (apo E) shift in TP2- or IgG-treated hamsters. Sera from hypercholesterolemic hamsters 0, 4, 11, and 14 d after the initial TP2 (A) or IgG (B) injection were run on fast-performance liquid chromatography, appropriate fractions were electrophoresed, and immunoreactive apo E was detected by enhanced chemiluminescence. Immunoblots were scanned by densitometry, the maximal adsorption of the peak fraction was normalized to one integrated optical density (IOD) unit, and the apo E band from the other fractions on the same immunoblot were expressed relative to the peak fraction. See Figure 1 for company sources.

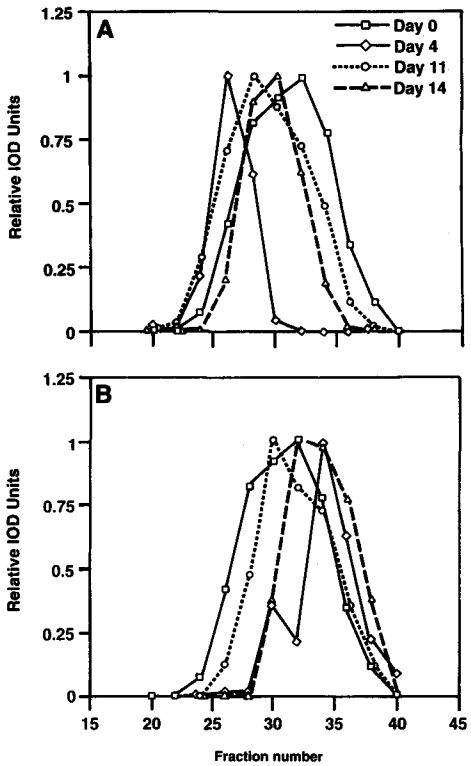


FIG. 3. Kinetics of cholesteryl ester transfer protein (CETP) shift in TP2- or IgG-treated hamsters. Nitrocellulose filters from the TP2- or IgG-injected hypercholesterolemic hamsters (A and B, respectively) were reprobed for CETP, and the resulting bands were quantitated by densitometry. The peak fraction at each time point was normalized to 1 IOD unit, and the CETP band from the other fractions on the same immunoblot was expressed relative to the peak fraction. See Figure 1 for company sources. See Figure 2 for abbreviation.

immunodetected CETP and a shift toward larger particles with maximal immunoreactivity localized to fractions 26–28 (Table 1). A similar profile was observed at 96 h with no further increases in CETP detected. However, in contrast to the persistence of apo E on larger particles, the CETP profile across the fractions by day 14 was similar to the day 0 pattern. A slight shift in immunodetected CETP was also detected in the IgG-treated controls, but this did not vary more than two fractions from the day 0 peak at fraction 32 (Fig. 3B). While quantitative changes in apoproteins were apparent by 24 h and showed no further increase for CETP at 4 d, apo E continued to increase. As with apo E, the increase in CETP appeared to be associated with lipoprotein particles which were intermediate in size between the LDL and HDL peaks. Therefore, while the accumulation of cholesterol-containing lipoprotein particles continued to increase during the first 7 d following CETP inhibition, the shifts in apo E and CETP distribution were apparent by 24 h. Expression of the IOD in the peak fraction in terms of cholesterol composition demonstrated a 17-fold increase in the amount of apo E when comparing day 0 to day 4, and a 26-fold increase in CETP. In a second series of hypercholesterolemic hamsters, normalization of the IOD-to-cholesterol content revealed a 23-fold increase in apo E between days 0 and 4 (data not shown). Therefore, inhibition of transfer activity in hypercholesterolemic hamsters was associated with both qualitative changes in lipoprotein size as well as significant quantitative changes in both apo E and CETP content. However, the relative distribution of apo E and CETP on these large HDL particles throughout the 14-d time course suggested distinct patterns of expression.

DISCUSSION

The lipoprotein changes associated with CETP deficiency have been well characterized following studies of patients with homozygous and heterozygous mutations of the CETP gene (3,4,10–12). The increase in HDL cholesterol and the appearance of larger HDL particles with a shift toward an increase in the HDL_2/HDL_3 ratio would suggest a lipoprotein profile less prone toward atherosclerosis. Clearly, the lipoprotein profile in CETP-deficient patients suggests that pharmacologic inhibition of CETP activity in humans may represent a therapeutic approach for increasing HDL cholesterol.

Inhibition of CETP activity in normocholesterolemic rabbits (5,6) and hypercholesterolemic hamsters (7) with monoclonal or polyclonal antibodies has been reported to increase HDL cholesterol. Studies in normocholesterolemic rabbits infused with a polyclonal CETP antiserum reported that while elevations in HDL cholesterol were observed, the only apoprotein change was an association of serum amyloid A protein with HDL (5). In rabbits injected with a neutralizing monoclonal antibody, TP1 had increased HDL cholesterol ester, a reduction in HDL triglyceride, a decrease in the clearance rate of [3H]cholesteryl ester-labeled HDL, and yet there was no significant change in HDL protein (6). This decrease in the clearance of cholesteryl ester-labeled HDL was consistent with the lower catabolic rates reported for apo A-I particles in CETP-deficient patients (26).

In previous studies, the inhibition of CETP activity in hamsters was associated with an increase in HDL cholesterol, the accumulation of large HDL and, in hypercholesterolemic animals, a shift in the distribution of both apo E and CETP onto larger HDL particles 7 d after *in vivo* neutralization of CETP (7). In the present study, the changes in apoprotein distribution were evaluated 24 h post-antibody injection and were followed throughout the entire two-week duration of the experiment. Inhibition of transfer activity resulted in an increase in apo E-containing lipoproteins with a size distribution between the HDL and LDL peaks. The increase in apo E was apparent by 24 h as was the shift to larger particles. This distribution of apo E persisted throughout the entire time course and was still evident when CETP activity and cholesterol profiles were similar to untreated or IgG-injected hamsters. Increased amounts of CETP were also detected in hamsters in which CETP had been inhibited and the distribution was toward larger HDL particles, as seen with apo E. However, in contrast to apo E, as transfer activity approached pretreatment levels by day 14, the distribution of CETP across the lipoprotein fractions also returned toward normal. In addition to the shifts in apo E and CETP to larger lipoproteins, the amount of these apoproteins increased more than 15-fold by day 4 when compared to the day 0 peak fraction.

The appearance of large apo E particles was previously reported in CETP-deficient subjects (3,4), although this was not apparent in the earlier rabbit studies. The increase in CETP activity associated with acceptor particles containing apo E (27) suggests that apo E may play a role in facilitating cholesteryl ester transfer when a significant fraction of CETP activity has been inhibited.

In vitro studies with CETP-neutralizing monoclonal antibodies have demonstrated an increase in apo A-IV and the appearance of apo E on larger HDL particles (28). Additional *in vitro* studies with Fab fragments also demonstrated that TP2 increased the binding of CETP to plasma lipoproteins and phospholipid vesicles (29). The relationship between the increased appearance and shift in CETP distribution *in vivo* and the effects of TP2 on CETP–lipoprotein association *in vitro* remains to be determined. In summary, the hypercholesterolemic hamster offers investigators a small animal model in which changes in lipoprotein profile, apoprotein distribution, and lesion regression can be monitored following inhibition of CETP activity by antibodies or by smaller molecular weight entities.

ACKNOWLEDGMENT

The authors thank Dr. Chandrasekhar for his review of this manuscript.

REFERENCES

1. Tall, A.R. (1990) *J. Clin. Invest. 86*, 379–384.
2. Tall, A.R. (1993) *J. Lipid Res. 34*, 1255–1274.

3. Inazu, A., Brown, M.L., Hesler, C.B., Agellon, L.B., Koizumi, J., Takata, K., Maruhama, Y., Mabuchi, H., and Tall, A.R. (1990) *New England J. Med. 323*, 1234–1238.
4. Yamashita, S., Sprecher, D.L., Sakai, N., Matsuzawa, Y., Tarui, S., and Hui, D.Y. (1990) *J. Clin. Invest. 86*, 688–695.
5. Abbey, M., and Calvert, G.D. (1989) *Biochim. Biophys. Acta 1003*, 20–29.
6. Whitlock, M.E., Swenson, T.L., Ramakrishnan, R., Leonard, M.T., Marcel, Y.L., Milne, R.W., and Tall, A.R. (1989) *J. Clin. Invest. 84*, 129–137.
7. Evans, G.F., Bensch, W.R., Apelgren, L.D., Bailey, D., Kauffman, R.F., Bumol, T.F., and Zuckerman, S.H. (1994) *J. Lipid Res. 35*,1634–1645.
8. Agellon, L.B., Walsh, A., Hayek, T., Moulin, P., Jiang, X.C., Shelanski, S.A., Breslow, J.L., and Tall, A.R. (1991) *J. Biol. Chem. 266*, 10796–10801.
9. Marotti, K.R., Castle, C.K., Boyle, T.P., Lin, A.H., Murray, R.W., and Melchior, G.W. (1993) *Nature 364*, 73–75.
10. Koizumi, J., Mabuchi, H.,Yoshimura, A., Michishita, I., Takeda, M., Itoh, H., Sakai, Y., Sakai, T., Ueda, K., and Takeda, R. (1985) *Atherosclerosis 58*, 175–186.
11. Brown, M.L., Inazu, A., Hesler, C.B., Agellon, L.B., Mann, C., Whitlock, M.E., Marcel, Y.L., Milne, R.W., Koizumi, J., Mabuchi, H., Takeda, R., and Tall, A.R. (1989) *Nature 342*, 448–451.
12. Sakai, N., Matsuzawa,Y., Hirano, K.-I., Yamashita, S., Nozaki, S., Ueyama, Y., Kubo, M., and Tarui, S. (1991) *Arterioscler. Thromb. 11*, 71–79.
13. Tall, A., Granot, E., Brocia, R., Tabas, I., Hesler, C., Williams, K., and Denke, M. (1987) *J. Clin. Invest. 79*, 1217–1225.
14. McPherson, R., Mann, C.J., Tall, A.R., Hogue, M., Martin, L., Milne, R.W., and Marcel, Y.L. (1991) *Arterioscler. Thromb. 11*, 797–804.
15. Moulin, P., Appel, G.B., Ginsberg, H.N., and Tall, A.R. (1992) *J. Lipid Res. 33*, 1817–1822.
16. Quinet, E.M., Huerta, P., Nancoo, D., Tall, A.R., Marcel, Y.L., and McPherson, R. (1993) *J. Lipid Res. 34*, 845–852.
17. Lagrost, L., Gandjini, G., Athias, A., Guyard-Dangremont, V., Lallemant, C., and Gambert, P. (1993) *Arterioscler. Thromb. 13*, 815–825.
18. Sicart, R., Sable-Amplis, R., and Guiro, A. (1984) *Comp. Biochem. Physiol. 78A*, 511–514.
19. Spady, D.K., and Dietschy, J.M. (1988) *J. Clin. Invest. 81*, 300–309.
20. Spady, D.K., and Dietschy, J.M. (1985) *Proc. Natl. Acad. Sci. USA. 82*, 4526–4530.
21. Stein, Y., Dabach, Y., Hollander, G., and Stein, O. (1990) *Biochim. Biophys. Acta 1042*, 138–141.
22. Quig, D.W., Arbeeny, C.M., and Zilversmit, D.B. (1991) *Biochim. Biophys. Acta 1083*, 257–264.
23. Hesler, C.B., Tall, A.R., Swenson, T.L., Weech, P.K., Marcel, Y.L., and Milne, R.W. (1988) *J. Biol. Chem. 263*, 5020–5023.
24. Tall, A., Sammett, D., and Granot, E. (1986) *J. Clin. Invest. 77*, 1163–1172.
25. Gerdes, L.U., Gerdes, C., Klausen, I.C., and Faergeman, O. (1992) *Clin. Chim. Acta 205*, 1–9.
26. Ikewaki, K., Rader, D.J., Sakamoto, T., Nishiwaki, M., Wakimoto, N., Schaefer, J.R., Ishikawa, T., Fairwell, T., Zech, L.A., Nakamura, H., Nagano, M., and Brewer, Jr., H.B. (1993) *J. Clin. Invest. 92*, 1650–1658.
27. Kinoshita, M., Arai, H., Fukasawa, M., Watanabe, T., Tsukamoto, K., Hashimoto, Y., Inoue, K., Kurokawa, K., and Teramoto, T. (1993) *J. Lipid Res. 34*, 261–268.
28. Bisgaier, C.L., Siebenkas, M.V., Hesler, C.B., Swenson, T.L., Blum, C.B., Marcel, Y.L., Milne, R.W., Glickman, R.M., and Tall, A.R. (1989) *J. Lipid Res. 30*, 1025–1031.
29. Swenson, T.L., Hesler, C.B., Brown, M.L., Quinet, E., Trotta, P.P., Haslanger, M.F., Gaeta, F.C.A., Marcel, Y.L., Milne, R.W., and Tall, A.R. (1989) *J. Biol. Chem. 264*,14318–14326.

[Received September 13, 1994; and in revised form January 27, 1995; Revision accepted January 30, 1995]

Uptake by Macrophages of Low-Density Lipoprotein Damaged by Nitrogen Dioxide in Air

Kiyomi Kikugawa*, Masatoshi Beppu and Yutaka Okamoto

Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

ABSTRACT: In order to know whether nitrogen dioxide, an environmental and endogenous free radical toxin, can participate in the formation of atherosclerotic lesions, damage to low-density lipoprotein (LDL) by nitrogen dioxide and uptake of the damaged LDL by macrophages were investigated. A solution of LDL at pH 7.5 was exposed to an atmosphere of nitrogen dioxide (70 ppm) in air at 37°C for 5 or 10 h. Lipid oxidation was induced by the exposure as assessed by the formation of thiobarbituric acid reactive substances. Apolipoprotein B was covalently cross-linked *via* nondisulfide bonds. Fluorescence analysis showed that tryptophan residues were extensively decreased, and amino acid analysis indicated that the contents of histidine, lysine, and tyrosine residues were decreased by 30–40, 10–20, and 20–30%, respectively. Binding of LDL to thioglycolate-induced mouse peritoneal macrophages was markedly increased by the exposure as observed by the binding of mouse erythrocytes coated with LDL. The activity of LDL to convert macrophages into lipid-laden foam cells was also increased by the exposure. Modification of lysine residues of apo B with lipid oxidation products formed by the exposure may be responsible for the uptake by macrophages. The results suggest the possibility that exposure of LDL *in vivo* to nitrogen dioxide participates in the formation of atherosclerotic lesions.

Lipids 30, 313–320 (1995).

There are several lines of evidence showing that oxidative changes of low-density lipoprotein (LDL) are associated with the formation of atherosclerotic lesions (1–5). LDL can be oxidized by endothelial cells, smooth muscle cells, macrophages, and cupric ion. Macrophages take up oxidized LDL more efficiently than normal LDL through the scavenger receptor. The excessive uptake of the oxidized LDL results in accumulation of oxidized cholesterol esters within the cells and converts the cells into lipid-laden foam cells. The conversion of macrophages into foam cells has been considered to be a possible early event in the formation of atherosclerotic lesions.

*To whom correspondence should be addressed.

Abbreviations: apo B, Apolipoprotein B; BHT, butylated hydroxytoluene; DPBS, Dulbecco's phosphate buffered saline; LDL, low-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance.

Nitrogen dioxide is a free radical toxin in polluted urban air (usually at 0.02–0.2 ppm) and in fresh smog (up to 100 ppm) (6). Inhalation of exogenous nitrogen dioxide has been suggested to cause pulmonary edema, pulmonary fibrosis, bronchitis (7–9), and cancer (10,11). Diffusion of exogenous nitrogen dioxide through an alveolar membrane to blood is possible because exposure of rats to nitrogen dioxide in air significantly damages the components of red blood cells (12). Nitrogen dioxide is also formed endogenously from nitrogen monoxide, which is synthesized by endothelial cells and plays an important physiological role as an endothelial-derived relaxing factor (13). Thus, nitrogen monoxide can react with oxygen to form nitrogen dioxide (Eq. 1) (14), and with superoxide to form peroxynitrite which, in turn, produces nitrogen dioxide by protonation (pKa = 6.8) (Eq. 2) (15,16).

$$2NO + O_2 \rightarrow 2NO_2 \qquad [1]$$

$$\begin{aligned} &\qquad\qquad + H^+ \\ &NO + O_2^- \rightarrow ONOO^- \rightarrow ONOOH \\ &ONOOH + O_2^- + H^+ \rightarrow NO_2 + O_2 + H_2O \end{aligned} \qquad [2]$$

Several reactions of biomolecules with nitrogen dioxide have been demonstrated. Exposure of polyunsaturated fatty acids to nitrogen dioxide in air gives many compounds, including lipid oxidation products (17–23). Exposure of tyrosine results in the formation of 3-nitrotyrosine and dityrosine, and exposure of tryptophan results in the breakdown of its indole ring (24). Reaction of cysteine with nitrogen dioxide gives certain highly reactive radical species (25).

In the present study, we investigated damage to LDL by exposure to nitrogen dioxide in air and the subsequent binding and uptake of the damaged LDL by macrophages. It was found that LDL was damaged extensively by exposure to nitrogen dioxide, and the damaged LDL readily bound to macrophages and converted the cells into lipid-laden foam cells. From a toxicological point of view, the present results may have significant implications for the formation of atherosclerotic lesions.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Materials. Nitrogen dioxide in air was obtained from Nippon Sanso Ltd. (Tokyo, Japan). The concentration of nitrogen dioxide was determined each time according to the method of Saltzman (26,27) using a conversion coefficient of NO_2^-/NO_2 of 0.84, where the measured nitrite concentrations were divided by 0.84 to convert to the nitrogen dioxide concentrations. Thus, nitrogen dioxide in air was bubbled into 10 mL of a solution containing sulfanilic acid, *N*-(1-naphthyl)ethylenediamine and acetic acid placed in a tube (15 mm i.d. × 105 mm) at a rate of 10 mL/min for 10 min. The solution was kept at room temperature for 15 min, and its absorbance at 545 nm was measured. The concentration of nitrite was determined by comparing the absorbance with that of the solution (10 mL) containing 0.375 μmole sodium nitrite. The concentration of nitrogen dioxide in air used in the present experiments was found to be 70 ppm.

Thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-Nitrotyrosine was obtained from Sigma Chemical Company (St. Louis, MO). Dityrosine was prepared as previously reported (28).

Analysis. Ultraviolet absorption spectra were measured on a Hitachi (Tokyo, Japan) U-2000 double beam spectrophotometer. Fluorescence spectra were measured on a Hitachi (Tokyo, Japan) 650-40 fluorescence spectrophotometer. The instrument was standardized with 0.1 μM quinine sulfate in 0.1 N sulfuric acid to give a fluorescence intensity of 100 at a maximum excitation/emission of 350/450 nm.

The TBA assay was performed by the colorimetric procedure according to the method of Ohkawa *et al.* (29) with modification (30). Briefly, a mixture of 0.80 mL of water containing sample, 50 μL of 0.8% BHT in glacial acetic acid, 0.20 mL of 8.1% SDS solution, 1.50 mL of 20% acetic acid solution adjusted to pH 3.5 with 10 N NaOH, and 1.50 mL of 0.8% TBA solution was kept at 5°C for 60 min and then heated at 100°C for 60 min. After cooling, the mixture was extracted with 1.0 mL of water and 5.0 mL of 1-butanol/pyridine (15:1, vol/vol). The absorbance at 532 nm of the organic phase (5.8 mL) was measured. The amount of thiobarbituric acid reactive substances (TBARS) was determined by the molar absorbance coefficient of the 1:2 malonaldehyde/TBA adduct, $\varepsilon = 156{,}000$. The amount of red pigment reflects not only malonaldehyde but alkenals and alkadienals, which are generated during lipid oxidation (30).

The concentration of proteins was determined by the Lowry method (31), using bovine serum albumin as a reference standard. SDS–polyacrylamide gel electrophoresis (PAGE) was performed using a 3.5% gel according to the method of Maguire *et al.* (32). The sample was treated with the sample buffer containing 40 mM dithiothreitol at 37°C for 1 h before electrophoresis, in order to destroy disulfide bonds in proteins. Protein bands were visualized by Coomassie blue.

Amino acid analysis was carried out on a Hitachi L-8500 high-performance amino acid analyzer equipped with a No. 2620 ion exchanger column (4.6 mm i.d. × 80 mm). The sample was hydrolyzed in 6 M HCl containing 4% thioglycolic acid at 110°C for 24 h under vacuum, and the mixture was evaporated to dryness. For the analysis of cysteine plus cystine and methionine, the sample was oxidized with performic acid in the usual manner prior to the hydrolysis. The column was eluted stepwise with sodium citrate buffer systems at pH 3.3, 4.0, 4.9, and alkaline pH, which are generally used for the analysis of amino acids and amino sugars. Common amino acids and 3-nitrotyrosine were detected by ninhydrin coloration at 570 nm (for most amino acids) and 440 nm (for proline), and fluorescent dityrosine was detected by fluorescence at 285/410 nm. 3-Nitrotyrosine and dityrosine appeared at retention times of 57 and 67 min, respectively.

LDL. Blood from a healthy human donor was collected into a citrate–phosphate–dextrose solution. Plasma was obtained by centrifugation and stored at −20°C for use within seven days. LDL was obtained from the plasma by preparative ultracentrifugation according to the method of Hatch and Lees (33) as described previously (34). In brief, aliquots of 6.5 mL of plasma mixed with EDTA at 1 mM were measured into ultracentrifuge tubes, and 3.25 mL of the density 1.006 solution was layered over the surface. The tubes were centrifuged at 31,000 × *g* and 16°C for 30 min. The bottom 6.5 mL layer was again overlaid with 3.25 mL of the density 1.006 solution, and the tubes were centrifuged at 115,000 × *g* for 6 h. Into the bottom 6.5 mL layer was mixed 3.25 mL of the density of 1.182 solution, and the tubes were centrifuged at 115,000 × *g* for 8 h. The top 2 mL layers from two tubes were combined and mixed with diisopropylfluorophosphate at 1 mM and sodium azide at 0.01% and finally with 4 mL of the density 1.182 solution. The density 1.006 solution (2.0 mL) was layered over the surface. The tube was centrifuged at 115,000 × *g* for 6 h. The 3–4 mL layer at the top of the higher density solution was collected. The LDL fraction was dialyzed at 4°C against phosphate buffered saline (PBS) containing 10 mM sodium phosphate buffer (pH 7.4) and 0.14 M sodium chloride, which had been freed from dissolved oxygen by purging with nitrogen gas. The fraction showed a single protein band in SDS–PAGE. Twenty milliliters of LDL solution in the buffer containing 23 mg protein were obtained and stored at 4°C for use within a day.

Macrophages. Macrophages were obtained from the peritoneal cavity of 7 to 15 week-old ddY male mice four days after intraperitoneal injection of 2–3 mL of 3% thioglycolate medium (Difco Laboratories, Detroit, MI). The peritoneal exudate cells obtained were washed twice with Hanks' balanced salt solution by centrifugation at 80 × *g* and 4°C for 10 min. The cells were then resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20 mM HEPES (pH 7.2), 50 units/mL penicillin and 50 μg/mL streptomycin (RPMI–HEPES medium) at 0.85×10^6 cells/mL.

Oxidation of LDL with cupric ion. LDL was oxidized as previously described (35,36). LDL (2 mg protein) in 3 mL of PBS was incubated with and without 5 μM cupric sulfate at 37°C for 24 h. The reaction was stopped by addition of 0.05

mM BHT. The mixture was dialyzed against PBS free from oxygen, and centrifuged to remove a small amount of precipitate.

Exposure of LDL to nitrogen dioxide. Into an L-shaped tube (content: 34 mL, 10 mm i.d. × 100 mm) was placed 5 mL of a solution of LDL (0.5 mg protein/mL) in 0.1 M phosphate buffer (pH 7.5), which had been prepared by dialyzing the LDL preparation against the buffer. Nitrogen dioxide in air was blown over the surface of the solution slightly swirled at a flow rate of 15 mL/min at 37°C for 5 and 10 h. As a control, purified air was similarly blown over the surface of the LDL solution. After dialysis against saline, the LDL solution was centrifuged to remove a small amount of precipitate.

The TBA assay was performed before and after dialysis. Fluorescence of the solution was measured, and SDS–PAGE of the solution was performed after dialysis. For the analysis of amino acid composition, the apolipoprotein B (apo B) fraction was isolated by delipidation according to the method previously reported (36). In brief, to one volume of the LDL solution, one volume of cold methanol and two volumes of cold chloroform were added. The mixture was shaken well and then centrifuged at 550 × *g* for 10 min. The aqueous layer and the turbid interphase were collected and combined, and dialyzed against water. The solution was lyophilized to obtain the apo B fraction. The fraction was dissolved into 0.3–0.6 mL of 1% SDS solution, and the aliquot containing 500 μg of protein was hydrolyzed and evaporated. The residue was redissolved into 0.50 mL of 0.02 M hydrochloric acid, and 10 and 100 μL of the solution were used for the analysis of common amino acids and dityrosine, respectively.

Erythrocytes coated with LDL samples. Blood was collected from ddY mice by cardiac puncture using acid citrate–dextrose preservative solution, and erythrocytes were centrifuged down, washed three times with Dulbecco's PBS (DPBS) without Ca^{2+} and Mg^{2+} (Ca^{2+}, Mg^{2+}-free DPBS) for removal of the buffy coats and plasma, and resuspended in isotonic saline to make a 4% cell suspension.

Coating of erythrocytes with LDL samples was performed as follows: A 4% erythrocyte suspension was mixed with an equal volume of a solution of the LDL sample (20 μg protein/mL) and incubated at 37°C for 15 min with occasional mixing. The mixture was centrifuged at 170 × *g* and 4°C for 15 min. The cell pellet was washed twice with the same buffer at 5°C by the centrifugation, and resuspended in RPMI–HEPES medium to make a 2% cell suspension. This operation was done on the day the coated erythrocytes were used. In this procedure, cupric ion-oxidized LDL samples were effectively adherent to the erythrocytes (1–3 ng LDL protein/10^6 cells) (37).

Binding of erythrocytes coated with LDL samples to macrophages. A 0.2 mL aliquot of the macrophage suspension was loaded onto a round cover glass (18-mm diameter). After incubation at 37°C for 1 h in a CO_2 incubator (5% CO_2/95% air), nonadherent cells were removed by washing three times with Ca^{2+}, Mg^{2+}-free DPBS, and adherent cell monolayers were subjected to the binding assay.

The suspension of erythrocytes coated with LDL sample (200 μL) was loaded on the macrophage monolayer. After incubation at 37°C for 1 h in a CO_2 incubator, nonadhering erythrocytes were removed by gentle washing with DPBS, and the cells were fixed with 1.25% glutaraldehyde in Ca^{2+}, Mg^{2+}-free DPBS. The number of the macrophages binding three or more erythrocytes on their surface was scored for the random fields of the cover glass under phase-contrast microscopy. At least 200 macrophages were examined for binding of coated erythrocytes, and the percentage of macrophages binding three or more erythrocytes were determined. The results were expressed as the mean ± SD of the triplicate experiments.

Foam cell formation. A 0.14 mL aliquot of the macrophage suspension was loaded onto a round cover glass (16-mm diameter). After incubation at 37°C for 1 h in a CO_2 incubator, nonadherent cells were removed by washing three times with Ca^{2+}, Mg^{2+}-free DPBS. The monolayer was placed in a well of a plastic 24-well plate (Corning), and to this was added 0.5 mL of RPMI 1640 medium containing LDL (100 μg protein/mL) and 5% fetal calf serum. The monolayer was incubated at 37°C for 24 h in a CO_2 incubator. After washing gently with Ca^{2+}, Mg^{2+}-free DPBS, the cells were fixed with 10% formalin for 60 min and washed with water. The oil droplets in the foam cells were stained by incubation with an Oil-red-O staining solution at 40–45°C for 30 min. After washing with water, the monolayer was subjected to the counter staining using Mayer's hematoxylin solution. After washing with water, the number of the macrophages containing oil droplets stained red was scored for the random fields of the cover glass under microscopy. At least 200 macrophages were examined. The results were expressed as the mean ± SD of the triplicate experiments.

RESULTS

Nitrogen dioxide-induced LDL damage. A surface of the solution of LDL at pH 7.5 and 37°C was exposed to nitrogen dioxide (70 ppm) in air for 5 and 10 h. As a control, a surface of the solution of LDL was similarly exposed to air. Extent of lipid oxidation was assessed by the formation of TBARS. While the amount of TBARS in the LDL solution was increased only slightly by the control air-exposure, it was extensively increased by the nitrogen dioxide exposure (Fig. 1). It has been clearly demonstrated that methyl linoleate and linolenate are oxidized into their hydroperoxide forms by exposure to nitrogen dioxide under the similar conditions (23), so TBARS provides an index of increased lipid oxidation of LDL particles. Hence, the results indicate that the lipid fraction of LDL particles was extensively oxidized by the nitrogen dioxide exposure. Substantial amount (70%) of TBARS disappeared after dialysis of the solution, probably because of loss of water-soluble low-molecular weight TBARS that were not bound to the LDL particles (data not shown).

The characteristic fluorescence due to tryptophan residues with maxima at 285/345 nm was decreased by both control

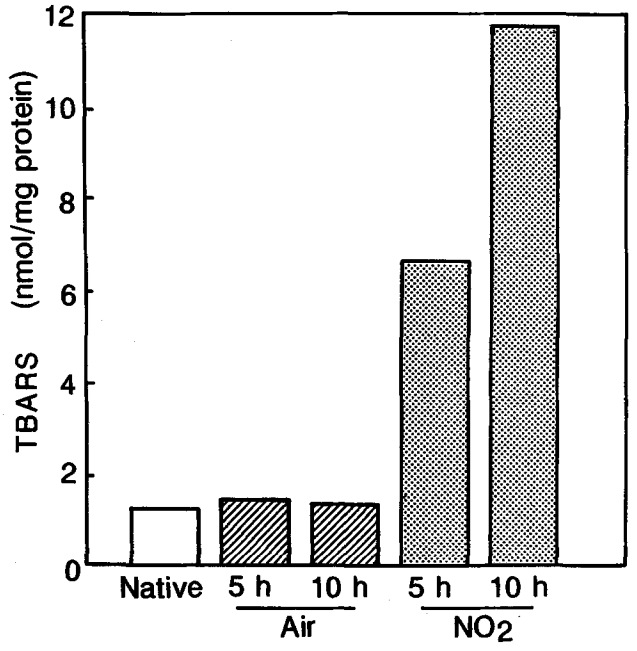


FIG. 1. The thiobarbituric acid reactive substances (TBARS) in the non-dialyzed low-density lipoprotein solution exposed to air and nitrogen dioxide (70 ppm) in air.

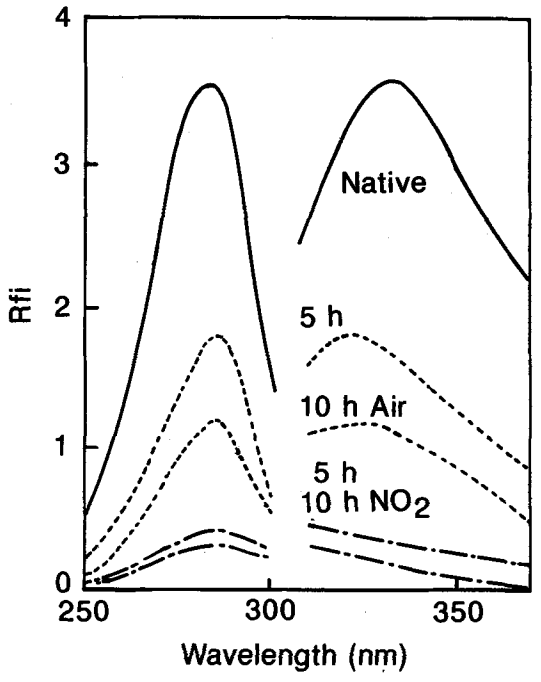


FIG. 2. Fluorescence spectra of the low-density lipoprotein solution, not exposed (—), exposed to air (---), and exposed to nitrogen dioxide (70 ppm) in air (–·–). Fluorescence spectra of the dialyzed solution containing 1 mg/mL protein were measured, and expressed as relative fluorescence intensity (Rfi) against 0.1 μM quinine sulfate in 0.1 N sulfuric acid.

air and nitrogen dioxide exposure, but the extent of the decrease by the nitrogen dioxide exposure was much greater than that by the air exposure (Fig. 2). SDS–PAGE was performed in the presence of dithiothreitol in order to detect apo B protein covalently cross-linked by nondisulfide bonds (Fig.

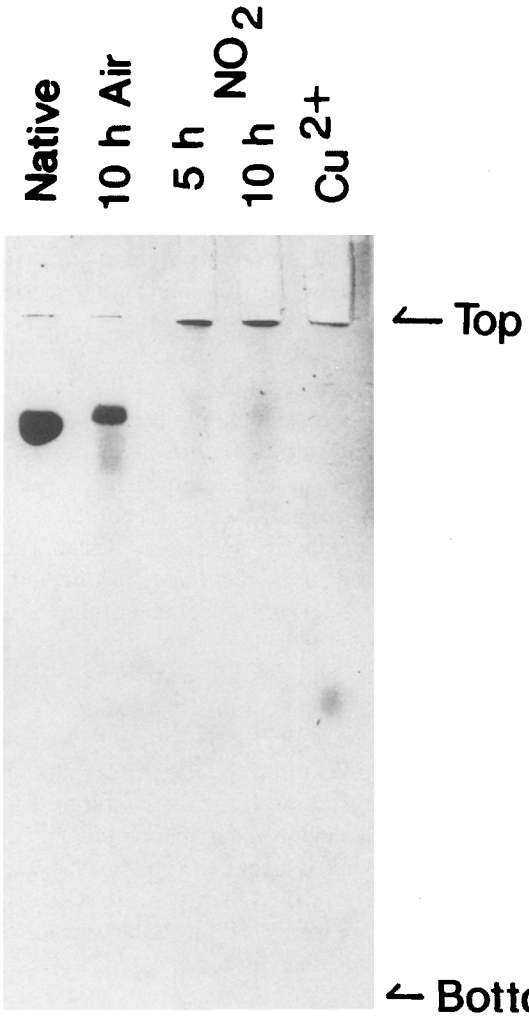


FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reduced conditions of the dialyzed low-density lipoprotein solution exposed to air and to nitrogen dioxide (70 ppm) in air. An aliquot containing 20 μg protein was subjected to the electrophoresis in the presence of dithiothreitol.

3). Apo B from LDL exposed to nitrogen dioxide was extensively cross-linked by nondisulfide covalent bonds, whereas apo B from LDL exposed to air was not. In contrast, apo B from the cupric ion-oxidized LDL was fragmented, as has been reported previously (38).

The apo B fractions obtained by delipidation of native LDL, LDL exposed to air, and LDL exposed to nitrogen dioxide in air were hydrolyzed and subjected to amino acid analysis to detect common amino acids and 3-nitrotyrosine by ninhydrin coloration and to detect dityrosine by fluorescence. The results of amino acid analysis are summarized in Table 1. In the apo B fractions from LDL exposed to air for 5 and 10 h, only histidine residues were significantly decreased as compared with the histidine content of the apo B fraction of native LDL. While the amount of tryptophan residues of apo B is too low to be detected by amino acid analysis, the residues may be partially destroyed, as observed by the fluorescence analysis (Fig. 2). Hence, histidine and tryptophan residues of apo B may be damaged by the air exposure. In the

TABLE 1
Amino Acid Composition of the Apoprotein B (apo B) Fraction of Low-Density Lipoprotein (LDL), Native, Exposed to Air, and Exposed to Nitrogen Dioxide (70 ppm) in Air

Amino acid	Amino acid residue[a] (mole)/mole of apo B from:					
	Native LDL		LDL exposed to air		LDL exposed to nitrogen dioxide	
	lit[b]		5 h	10 h	5 h	10 h
Alanine	310	334	326	328	327	332
Arginine	172	167	170	165	165	165
Aspartic acid	536	535	525	524	574	506
Cysteine + cystine	—	47	38	35	27	37
Glycine	248	269	280	287	281	292
Glutamic acid	626	607	613	615	622	632
Histidine	126	131	107	104	83	82
Isoleucine	294	228	244	246	230	241
Leucine	594	535	534	530	485	536
Lysine	402	409	384	381	359	348
Methionine	88	102	97	96	99	100
Phenylalanine	252	235	237	235	228	232
Proline	206	209	207	207	221	213
Serine	386	419	438	445	431	454
Threonine	320	307	318	319	315	322
Tryptophan	—	—	—	—	—	—
Tyrosine	168	173	173	173	122	126
3-Nitrotyrosine	0	0	0	0	0	0
Dityrosine $\times 10^3$	0	6	8	14	6	14
Valine	268	297	313	315	434	380
Total	4996	5004	5004	5005	5003	4998

[a]Amino acid residues calculated per mol apo B with 5000 residues.

[b]From Reference 39. Values underlined indicate those significantly decreased from those of native LDL.

apo B fractions from LDL exposed to nitrogen dioxide for 5 and 10 h, histidine, lysine, and tyrosine residues were decreased. Histidine residues were decreased by 30–40%, lysine residues by 10–20%, and tyrosine residues by 20–30%. Tryptophan residues must be extensively destroyed because fluorescence due to tryptophan residues was diminished (Fig. 2). Hence, histidine, lysine, tyrosine, and tryptophan residues of apo B were found to be susceptible to the nitrogen dioxide exposure. It is interesting to note that 3-nitrotyrosine and dityrosine were not detectable in the apo B fractions from LDL exposed to nitrogen dioxide, whereas they are detectable in bovine serum albumin and α-crystallin exposed to nitrogen dioxide (24).

Macrophage adhesion of LDL exposed to nitrogen dioxide. The ability of LDL exposed to nitrogen dioxide to bind to the monolayer of thioglycolate-induced mouse peritoneal macrophages was examined. The binding ability of LDL to macrophages was measured by observing the binding of erythrocytes coated with LDL to macrophages microscopically (37). Figure 4 shows that the percentage of macrophages binding erythrocytes coated with native LDL was less than 20%, and the percent macrophage adhesion of the control LDL exposed to air for 5 and 10 h was about 33%. The percent macrophage adhesion increased with increasing exposure of LDL to nitrogen dioxide. The percent macrophage adhesion of LDL exposed to nitrogen dioxide for 5 h was 50%, and that of LDL exposed to nitrogen dioxide for 10 h was 83%. The latter value was comparable to that (73%) of the cupric ion-oxidized LDL, which is known to bind effectively to macrophages (35,36).

Furthermore, the number of erythrocytes coated with LDL exposed to nitrogen dioxide for 5 and 10 h that bound to one macrophage cell was much larger than that of erythrocytes coated with the control LDL exposed to air (data not shown). Hence, LDL exposed to nitrogen dioxide showed a high ability to bind to macrophages.

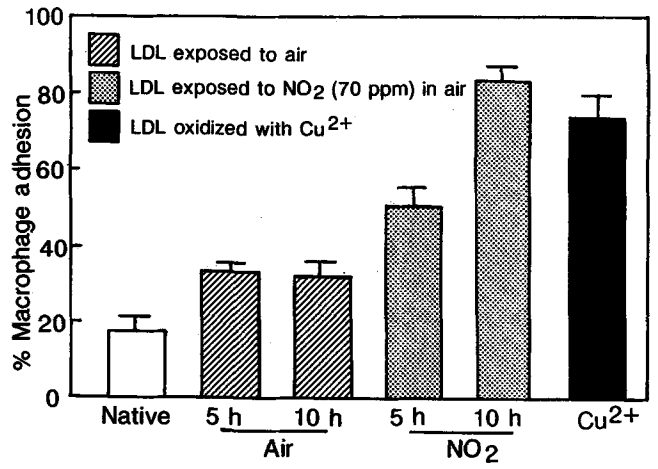


FIG. 4. Macrophage adhesion of mouse erythrocytes coated with low-density lipoprotein (LDL) exposed to air, exposed to nitrogen dioxide (70 ppm) in air, or oxidized with cupric ion. Thioglycolate-induced mouse peritoneal macrophages were used. The mean ± SD of percentage of cells with adherent erythrocytes in three experiments are shown.

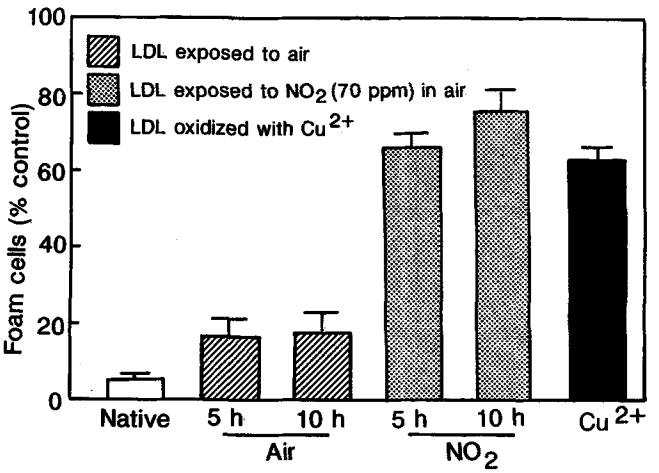


FIG. 5. Conversion of mouse peritoneal macrophages into lipid-laden foam cells by low-density lipoprotein (LDL) exposed to air, exposed to nitrogen dioxide (70 ppm) in air, or oxidized with cupric ion. The mean percentage of foam cells ± SD of three experiments are shown.

Conversion of macrophages into foam cells by LDL exposed to nitrogen dioxide. The ability of LDL exposed to nitrogen dioxide to convert thioglycolate-induced mouse peritoneal macrophages into lipid-laden foam cells was examined. Figure 5 shows that only 5% of the total macrophages were converted into foam cells in the presence of native LDL. The control LDL exposed to air for 5 and 10 h converted about 20% of the cells into foam cells. The LDL exposed to nitrogen dioxide for 5 and 10 h, however, converted a greater proportion of macrophages into foam cells. The ability of LDL exposed to nitrogen dioxide to convert macrophages into foam cells was comparable to that (65%) of the cupric ion-oxidized LDL. Hence, the ability of LDL to convert macrophages into foam cells was increased by the nitrogen dioxide exposure.

DISCUSSION

Plasma lipoprotein LDL is believed to play an important role in the formation of atherosclerotic lesions. Oxidation of LDL appears to increase its uptake into macrophages of the arterial wall and may thus convert macrophages into lipid-laden foam cells rich in oxidized cholesterol esters. This process has been considered to be a possible early event in the formation of atherosclerotic lesions (1–3). Oxidation of LDL *in vitro* by cupric ion has been characterized by the decrease in polyunsaturated fatty acids and the generation of lipid hydroperoxides and aldehydes (40). Apo B is fragmented (38) and becomes fluorescent by interaction with aldehydes generated during lipid oxidation (35,40). Lysine residues of apo B are modified by their interactions with lipid oxidation products (35,41,42). A decreased number of unaltered tryptophan residues has also been shown (43). The modification of lysine residues of apo B (42,44–48) has been considered to be the alteration of oxidized LDL that is responsible for their enhanced uptake by macrophages. On the basis of experiments conducted with cultured cells, it has been suggested that a number of oxidants are involved in damaging LDL. Superoxide (49), the products of lipoxygenase (50), and certain radical species generated by interaction of nitrogen monoxide with superoxide (51) have been suggested as possible oxidants.

In the present experiment, it was found that exposure of LDL to nitrogen dioxide (70 ppm) in air resulted in lipid oxidation as assessed by TBARS, apo B cross-linking by nondisulfide covalent bonds, and marked decreases of histidine, lysine, tyrosine, and tryptophan residues of apo B. Our present findings suggest that modification of apo B is derived not only from the reaction with secondary lipid oxidation products but also from the direct reaction with nitrogen dioxide.

Control exposure of LDL to air alone resulted in the loss of histidine and tryptophan residues of apo B. Although this exposure only slightly increased TBARS, lipid oxidation may have been induced because histidine and tryptophan residues of apo B, which are known to react readily with lipid oxidation products (35,42,43), were damaged. It has been shown that modification of delipidated apo B with lipid hydroperoxides or their aldehyde degradation products causes the loss of histidine, lysine, and tryptophan residues (42). The loss of histidine, lysine, and tryptophan in apo B of LDL exposed to nitrogen dioxide may be due to the increased lipid oxidation induced by nitrogen dioxide in air. This idea is supported by the fact that nitrogen dioxide in air at these low concentrations readily initiates lipid oxidation of polyunsaturated fatty acids (23). Nondisulfide cross-links produced in apo B by the nitrogen dioxide exposure may be due to the reaction of ly-

sine residues of apo B, with aldehydes resulting from lipid oxidation, as has been demonstrated previously (52).

It has been shown that exposure of proteins to nitrogen dioxide in air results in the loss of tyrosine and tryptophan residues of the proteins (24). The decrease of tyrosine residues in proteins exposed to nitrogen dioxide may be due to its radical attack to generate tyrosine phenoxy radical, which in turn is converted into 3-nitrotyrosine and dityrosine. The decrease of tryptophan residues may also be due to the radical reactions of nitrogen dioxide. The decrease in tyrosine residues of apo B induced by exposure to nitrogen dioxide may be due to the radical attack of nitrogen dioxide to form tyrosine phenoxy radical, but because we did not detect 3-nitrotyrosine and dityrosine in the treated apo B, the intermediary phenoxy radical may be converted into other species by interaction with lipid radicals being generated simultaneously.

The ability of LDL to bind to macrophages was assayed by a newly devised method using erythrocytes, where the binding of LDL to macrophages was microscopically observed as the binding of erythrocytes coated with LDL (37). LDL exposed to nitrogen dioxide greatly increased erythrocyte binding to macrophages. Furthermore, LDL exposed to nitrogen dioxide acquired the ability to convert macrophages into lipid-laden foam cells. The results indicate that LDL exposed to nitrogen dioxide was recognized and readily taken up by macrophages and converted the cells into lipid-laden foam cells. The recognition site for macrophage uptake of LDL exposed to nitrogen dioxide may be the modified lysine residues of apo B with aldehydes generated by lipid oxidation, similar to that seen with cupric ion-oxidized LDL (42,44–48). Scavenger receptors of macrophages may play a role in the uptake of LDL exposed to nitrogen dioxide.

In conclusion, nitrogen dioxide in air can cause damage to apo B of LDL, making the lipoprotein susceptible to the uptake by macrophages. Environmental and endogenous nitrogen dioxide may be one of possible oxidants for the formation of atherosclerotic lesions.

REFERENCES

1. Henning, B., and Chow, C.K. (1988) *Free Rad. Biol. Med. 4*, 99–106.
2. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witztum, J.L. (1989) *New Engl. J. Med. 320*, 915–924.
3. Steinbrecher, U.P., Zhang, H., and Lougheed, M. (1990) *Free Rad. Biol. Med. 9*, 155–168.
4. Witztum, J.L., and Steinberg, D. (1991) *J. Clin. Invest. 88*, 1785–1792.
5. Esterbauer, H., Gebicki, J., Puhl, H., and Jergens, G. (1992) *Free Rad. Biol. Med. 13*, 341–390.
6. Pryor, A.W. (1992) *Free Rad. Biol. Med. 13*, 659–676.
7. Stephen, R.J., Freeman, G., and Evans, M. (1972) *Arch. Environ. Hlth. 24*, 160–179.
8. Guidotti, T.L. (1972) *Env. Res. 15*, 443–472.
9. Dawson, S.V., and Schenker, M.B. (1979) *Am. Rev. Resp. Dis. 120*, 281–292.
10. Richter, A., and Richter, V.A. (1983) *Arch. Environ. Hlth. 38*, 69–75.
11. Richter, A. (1988) *J. Tox. Environ. Hlth. 25*, 383–390.
12. Kaya, K., Miura, T., and Kubota, K. (1980) *Environ. Res. 23*, 397–409.
13. Palmer, R.M.J., Ferrige, A.G., and Moncada, S. (1987) *Nature 327*, 524–526.
14. Schwartz, S.E., and White, W.H. (1983) *Trace Atmospheric Constituents* (Schwartz, S.E., ed.) pp. 1–116, John Wiley & Sons, New York.
15. Radi, R., Beckman, J.S., Bush, K.M., and Freeman, B.A. (1991) *Arch. Biochem. Biophys. 288*, 481–487.
16. Koppenol, W.H., Morens, J.J., Pryor, W.A., Ishinoponlos, H., and Beckman, J.S. (1992) *Chem. Res. Toxicol. 5*, 834–842.
17. Thomas, H.V., Mueller, P.K., and Lyman, R.L. (1968) *Science 159*, 532–534.
18. Sagai, M., Ichinose, T., Oda, H., and Kubota, K. (1981) *Lipids 16*, 64–67.
19. Roehm, J.N., Hadley, J.G., and Menzel, D.B., *Arch. Environ. Hlth. 23*, 142–148.
20. Collard, R., and Pryor, W.A. (1979) *Lipids 14*, 748–751.
21. Pryor, W.A., and Lightsey, J.W. (1981) *Science 214*, 435–437.
22. Pryor, W.A., Lightsey, J.W., and Church, D.F. (1982) *J. Am. Chem. Soc. 104*, 6685–6692.
23. Kikugawa, K., and Kogi, K. (1987) *Chem. Pharm. Bull. 35*, 344–349.
24. Kikugawa, K., Kato, T., and Okamoto, Y. (1994) *Free Rad. Biol. Med. 16*, 373–382.
25. Kikugawa, K., Hiramoto, K., Okamoto, Y., and Hasegawa, Y. (1994) *Free Rad. Res. 21*, 399–408.
26. Saltzman, B.D. (1954) *Anal. Chem. 26*, 1949–1955.
27. Saltzman, B.D. (1960) *Anal. Chem. 32*, 135–136.
28. Kikugawa, K., Kato, T., Beppu, M., and Hayasaka, A. (1991) *Biochim. Biophys. Acta 1096*, 108–114.
29. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochim. 95*, 351–358.
30. Kosugi, H., Kojima, T., and Kikugawa, K. (1991) *J. Am. Oil Chem. Soc. 68*, 51–55.
31. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265–275.
32. Maguire, G.F., Lee, M., and Connelly, P.W. (1989) *J. Lipid Res. 30*, 757–761.
33. Hatch, F.T., and Lees, D.S. (1968) *Adv. Lipid Res. 6*, 1–68.
34. Kojima, T., Kikugawa, K., and Kosugi, H. (1990) *Chem. Pharm. Bull. 38*, 3414–3418.
35. Steinbrecher, U.P. (1987) *J. Biol. Chem. 262*, 3603–3608.
36. Parthasarathy, S., Fong, L.G., Otero, F.D., and Steinberg, D. (1987) *Proc. Natl. Acad. Sci. USA, 84*, 537–540.
37. Beppu, M., Hora, M., and Kikugawa, K. (1994) *Biol. Pharm. Bull. 17*, 39–46.
38. Schuh, J., Fairclough, G.F., Jr., and Haschemeyer, R.H. (1978) *Proc. Natl. Acad. Sci. USA 75*, 3173–3177.
39. Weisgraber, K.H., Innerarity, T.L., and Mahley, R.W. (1978) *J. Biol. Chem. 253*, 9053–9062.
40. Esterbauer, H., Jurgens, G., Quehenberger, O., and Koller, E. (1987) *J. Lipid Res. 28*, 495–509.
41. Steinbrecher, U.P., Witztum, J.L., Parthasarathy, S., and Steinberg, D. (1987) *Arteriosclerosis 7*, 135–143.
42. Alaiz, M., Beppu, M., Ohishi, K., and Kikugawa, K. (1994) *Biol. Pharm. Bull. 17*, 51–57.
43. Reyftmann, J.-P., Santus, R., Maziere, J.-C., Morliere, P., Salmon, S., Candide, C., Maziere, C., and Haigle, J. (1990) *Biochim. Biophys. Acta 1042*, 159–167.
44. Haberland, M.E., Fogelman, A.M., and Edwards, P.A. (1982) *Proc. Natl. Acad. Sci. USA 79*, 1712–1716.
45. Haberland, M.E., Olch, C.L., and Fogelman, A.M. (1984) *J. Biol. Chem. 259*, 11305–11311.
46. Jurgens, G., Lang, J., and Esterbauer, H. (1986) *Biochim. Biophys. Acta 875*, 103–114.

47. Steinbrecher, U.P., Lougheed, M., Kwan, W.-C., and Dirks, M. (1989) *J. Biol. Chem. 264*, 15216–15223.
48. Zhang, H., Yang, Y., and Steinbrecher, U.P. (1993) *J. Biol. Chem. 268*, 5535–5542.
49. Cathcart, M.K., McNally, A.K., Morel, D.W., and Chisolm, G. (1989) *J. Immunol. 142*, 1963–1969.
50. Yla-Herttuala, S., Rosenfield, M.E., Parthasarathy, S., Glass, C.K., Sigal, E., Witztum, J.L., and Steinberg, D. (1900) *Proc. Natl. Acad. Sci. USA 87*, 6959–6963.
51. Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T., and Moncada, S. (1992) *Free Rad. Res. Commun. 17*, 9–20.
52. Yamaki, S., Kato, T., and Kikugawa, K. (1992) Chem. Pharm. Bull. 40, 2138–2142.

[Received February 7, 1994, and in revised form January 23, 1995; Revision accepted February 10, 1995]

Antioxidant Activity of Thiocholesterol on Copper-Induced Oxidation of Low-Density Lipoprotein

Mitsuo Tanaka* and Mitsuo Nakagawa

Showa College of Pharmaceutical Sciences, Tokyo 194, Japan

ABSTRACT: The effect of thiocholesterol (SH-Chol) on the copper-induced *in vitro* oxidation of low-density lipoprotein (LDL; $1.019 < d < 1.063$) was investigated. Among the antioxidants tested, including cysteine, glutathione, 2-mercaptoethanol, dithiothreitol, probucol, thiopalmitic acid, and SH-Chol, SH-Chol was the most effective antioxidant in copper-induced LDL oxidation. Also, SH-Chol completely inhibited the formation of oxysterols, i.e., 7-hydroxycholesterol and 7-ketocholesterol, in LDL particles and reduced 1,1-diphenyl-2-picrylhydrazyl used as stable free-radical model. Moreover, SH-Chol suppressed the degradation of endogenous α-tocopherol in LDL particles. These findings indicate that SH-Chol acts as antioxidant in the oxidative damage of LDL *in vitro* and as a free-radical scavenger in lipid peroxidation.

Lipids 30, 321–325 (1995).

The oxidative modification of low-density lipoprotein (LDL) is believed to be related to foam cell formation *via* uptake by macrophage scavenger receptors and may play an important role in atherosclerotic processes (1–5). Oxidative modification of lipids can be catalyzed by small amounts of transition metal ions such as Cu^{2+} and Fe^{2+} as well as macrophages, smooth muscle, and endothelial cells (6–10). Since these metals, even in the absence of cells, mimic cell-induced changes in the lipoprotein (9,10), the transition metal ion-induced modifications of LDL and peroxidation of LDL-lipids is believed to be a useful model system for the study of atherogenesis (11,12). In addition, heavy metals such as mercury and cadmium also are capable of inducing lipid peroxidation (13,14). The enhanced lipid peroxidation may be related to the toxicities of these metals (15).

On the other hand, the presence of water-soluble and sulfur-containing antioxidants such as glutathione, 2-mercaptopropionylglycine and N-acetylcysteine can prevent free radical-mediated oxygen damage *in vivo* and *in vitro* (16–19). Similarly, besides the water-soluble, sulfur-containing antioxidants, we reported recently that thiopalmitic acid (SH-Pal), a lipid-soluble, sulfur-containing compound, acts as an antioxidant and free-radical scavenger on microsomal lipid peroxidation of rat liver (20). Another lipid-soluble, sulfur-containing compound thiocholesterol (SH-Chol) has been reported to protect against mercury and lead poisoning in mice (21,22).

Therefore, in this paper, we investigated whether SH-Chol and SH-Pal, as well as the water-soluble antioxidants mentioned above, can protect against the copper-induced oxidation of LDL *in vitro.*

MATERIALS AND METHODS

Chemicals. SH-Chol, 7-ketocholesterol (7-Keto Chol), α-tocopherol, probucol (4,4′-(isopropylidenedithio)*bis*[2,6-di-*tert*-butylphenol]), glutathione, dithiothreitol, 2-mercaptoethanol, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Company (St. Louis, MO). Thioacetic acid, palmitoyl chloride, propylamine, and anhydrous pyridine were from Aldrich Chemical Co. (Milwaukee, WI). 7-Hydroxycholesterol (7-OH Chol) was obtained from Midorijugi Co. (Tokyo, Japan). Thiobarbituric acid (TBA) was from Wako Pure Chemical Industries (Tokyo, Japan). All other chemicals used were of analytical grade.

Synthesis. SH-Pal was prepared by the method of Shin and Quinn (23) as described previously by (20). The SH-Pal synthesized was characterized by nuclear magnetic resonance (NMR; JEOL JNM-EX90, Tokyo, Japan) and infrared (IR; JASCO IR-700, Tokyo, Japan) spectroscopy and by gas chromatography/mass spectrometry (GC/MS; Hewlett-Packard HP5890A, Palo Alto, CA). The purity of SH-Pal was about 90%.

Preparation of lipoprotein. LDL was isolated from fresh normal human plasma by ultracentrifugation as described previously (24). Serum (4.0 mL) was added to ultracentrifuge tubes, and a $d = 1.006$ g/mL solution (2.0 mL) was layered

*To whom correspondence should be addressed at Showa College of Pharmaceutical Sciences, 3-3165, Higashi-tamagawagakuen, Machida, Tokyo 194, Japan.

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; IR, infrared; 7-Keto Chol, 7-ketocholesterol; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; 7-OH Chol, 7-hydroxycholesterol; SH-Chol, thiocholesterol; SH-Pal, thiopalmitic acid; TBARS, thiobarbituric acid reactive substances; Tris·HCl, tris(hydroxymethyl)-aminomethane hydrochloride; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

over the surface. The tube was capped and centrifuged in a Hitachi 65P ultracentrifuge (Hitachi Co., Tokyo, Japan) at 20,000 rpm for 30 min at 4°C. The upper layer (2.0 mL) containing chylomicrons was removed, and a d = 1.006 g/mL solution (2.0 mL) was layered over the surface. The tube was centrifuged at 40,000 rpm for 16 h at 4°C. The upper layer (2.0 mL) containing very low density lipoprotein (VLDL) was removed, and a d = 1.182 g/mL solution (2.0 mL) (final d = 1.063 g/mL) was added and mixed. Then, the mixture was centrifuged at 40,000 rpm for 20 h at 4°C. The upper layer (1.0 mL) containing LDL was removed through the side of the tube with a needle and syringe. The LDL preparation obtained was dialyzed exhaustively against 0.15 M NaCl/0.02M tris(hydroxy-methyl)aminomethane–HCl (Tris–HCl) buffer (pH 7.4) containing 0.02% EDTA at 4°C for 24 h. The LDL preparation containing EDTA was used within 2 wk. Prior to the LDL oxidation experiment, the EDTA was removed from the EDTA–LDL preparation by dialyzing against 0.1 5M NaCl/0.02M Tris–HCl buffer (pH 7.4) and sterilized by filtration (0.45 μm). The LDL preparation obtained was used within 24 h after EDTA removal. Oxidized LDL was prepared by incubation of LDL (200 μg protein/mL 0.15 M NaCl/0.02 M Tris–HCl buffer, pH 7.4) with 10 μM $CuSO_4$.

Assay of lipid peroxidation products. The formation of thiobarbituric acid reactive substances (TBARS) was used as an indicator of lipid peroxidation. The lipid peroxide content of oxidized LDL was measured by a modified method of the TBARS assay as described previously (25). TBARS values are expressed as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as a standard.

Measurement of sterols. The content of sterols (oxysterol and SH-Chol) in LDL was determined by capillary GC/MS as we have described previously (26). The sterols in LDL were extracted with hexane/isopropanol (3:1, vol/vol), and the organic phase was evaporated to dryness under nitrogen. The extracts were dissolved in a small volume of toluene and injected directly into the capillary gas chromatograph/mass spectrometer.

Determination of α-tocopherol. The determination of α-tocopherol content was carried out by high-performance liquid chromatography (HPLC; Hitachi D-6100) with fluorometric detector according to partially modified procedures of the method of Bieri *et al.* (27). All samples for HPLC analysis were extracted with hexane/isopropanol (3:1, vol/vol). The organic phase was evaporated to dryness under nitrogen. The extracts were dissolved in a small volume of ethanol and then applied to the HPLC instrument equipped with Inertsil ODS-2 column (5 mm × 150 mm; Gasukuro Kogyo Co., Tokyo, Japan).

Reduction of DPPH. The reduction of DPPH free radical was measured by the method of Uchiyama *et al.* (28). Reaction mixtures (final volume 5.0 mL) contained 1.0 mL of 0.5 mM DPPH–ethanol solution, 2.0 mL of ethanol, and 2.0 mL of 0.1 M sodium acetate buffer (pH 5.5). In the case of the addition of SH-Chol, α-tocopherol, and cysteine to the reaction mixture, SH-Chol and α-tocopherol were added in 2.0 mL of ethanol, and cysteine was added in 2.0 mL of acetate buffer (pH 5.5). The incubation was carried out at 37°C for 30 min. The reduction of DPPH free radicals was measured with the optical density at 517 nm.

Determination of enzyme protein content. The protein content was determined by the method of Lowry *et al.* (29) using crystalline bovine serum albumin as a standard.

Statistical evaluation. All data were expressed as the mean ± SD. Statistical analysis was performed using a one-way analysis of variance and Scheffe's method for multiple comparisons.

RESULTS AND DISCUSSION

In order to investigate the protective potencies of various antioxidants on copper-induced oxidation of LDL, LDL with 10 μM $CuSO_4$ was incubated with cysteine (100 μM), glutathione (100 μM), 2-mercaptoethanol (100 μM), dithiothreitol (100 μM), probucol (50 μM), SH-Pal (90 μM), and SH-Chol (30 μM). As shown in Figure 1, under the present experimental conditions, cysteine, 2-mercaptoethanol, dithiothreitol, probucol, SH-Pal, and SH-Chol protected against

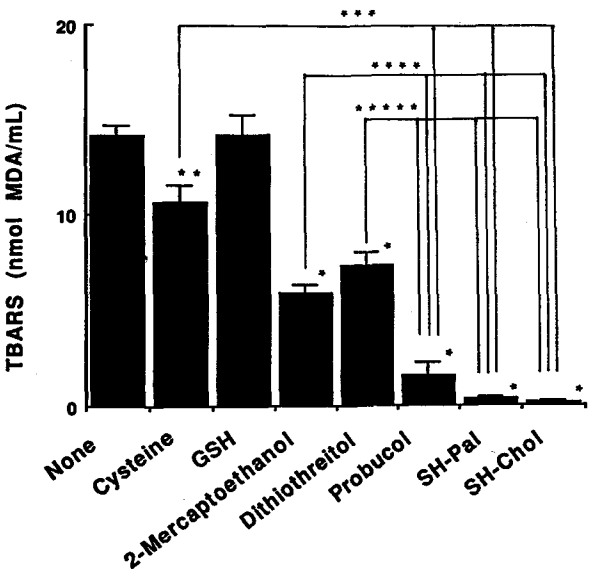


FIG. 1. Effect of various antioxidants on the copper-catalyzed oxidation of low-density lipoprotein (LDL). The incubation mixture contained LDL (200 μg/mL), 10 μM $CuSO_4$, and 0.15 M NaCl/0.02 M Tris-HCl buffer (pH 7.4) in a total volume of 1.0 mL. Probucol, thiocholesterol (SH-Chol), and thiopalmitic acid (SH-Pal) in the standard incubation mixture were added as ethanol solution (10 μL). Incubations were carried out at 37°C for 4 h. The values are the means ± SD of three separate experiments (triplicates for each experiment) done on the same LDL sample. Antioxidant concentrations: cysteine (100 μM); glutathione (GSH) (100 μM); 2-mercaptoethanol (100 μM); dithiothreitol (100 μM); probucol (50 μM); SH-Pal (90 μM); SH-Chol (30 μM). $^{*}P < 0.001$; $^{**}P < 0.01$ from the value obtained by addition of Cu^{2+} alone; $^{***}P < 0.001$ from cysteine; $^{****}P < 0.01$ from 2-mercaptoethanol; $^{*****}P < 0.001$ from dithiothreitol all by one-way analysis of variance with Scheffe's test. TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

copper-induced oxidation of LDL as measured by the amount of TBARS. Upon addition of 100 μM of probucol, SH-Pal, and SH-Chol to the Cu^{2+}–LDL incubation medium, the amount of TBARS generated was scarcely detectable (data not shown). Accordingly, the protective action of cysteine, 2-mercaptoethanol, and dithiothreitol was significantly lower than that of probucol, SH-Pal, and SH-Chol (vs. cysteine and dithiothreitol, $P < 0.001$, vs. 2-mercaptoethanol, $P < 0.01$, respectively), which are lipid-soluble antioxidants. Notably, SH-Chol appears to be the most effective antioxidant during copper-induced oxidation of LDL. The antioxidant action may be partly due to the protection from copper attack on the LDL by the association between LDL and SH-Chol rather than the direct interaction between copper and SH-Chol.

We next investigated the effect of SH-Chol concentration on copper-induced oxidation of LDL *in vitro*. As shown in Table 1, when LDL was incubated with 10 μM $CuSO_4$ at 37°C for 4 h, the oxidative modification of LDL was significantly increased. Upon addition of increasing amounts of SH-Chol to the incubation medium, the oxidation of LDL was effectively suppressed. In particular, upon addition of over 60 μM SH-Chol, TBARS were below the control level. However, when SH-Chol (60 μM) was added to the medium at the end of incubation period, the suppressive action of SH-Chol on LDL oxidation was no longer observed, demonstrating that SH-Chol did not interfere with the measurement of TBARS.

We next investigated the time-course of LDL oxidation by copper ions *in vitro*. As shown in Figure 2, the time-course of LDL oxidation showed an initial lag phase of 1 h, followed by a propagation phase over the next 1 h. The addition of SH-Chol (15 μM) at 0 h (Fig. 2a) or at 0 and 1 h (Fig. 2b) delayed the onset of the propagation phase. The addition of SH-Chol at 0 and 2 h (Fig. 2c) was less effective than its addition at 0 and 1 h (Fig. 2b), presumably because SH-Chol reacts with the lipid peroxyl radicals already formed in LDL and present in the incubation medium. In fact, as shown in Table 2,

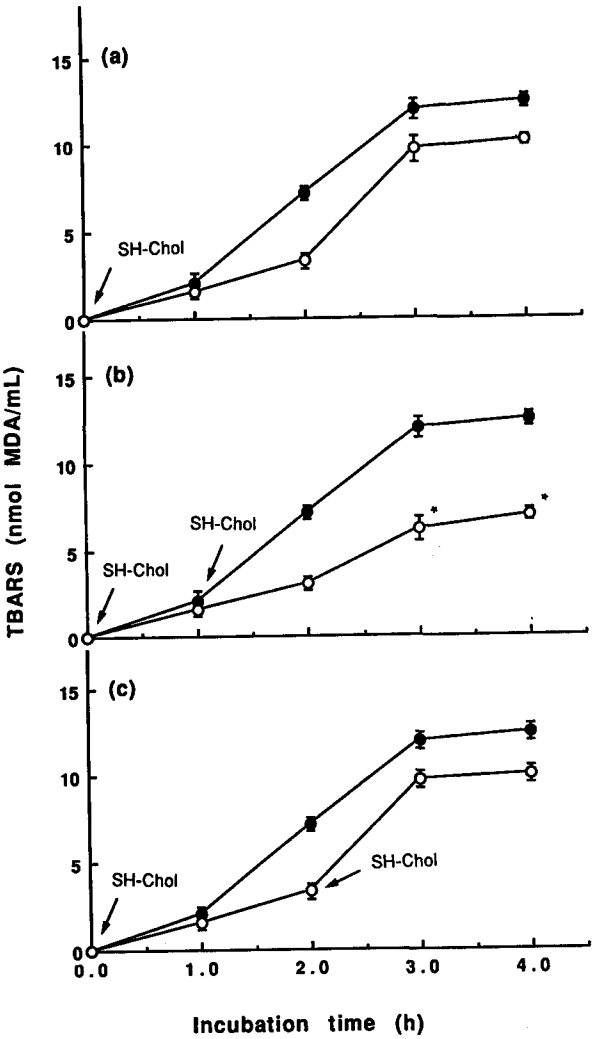


FIG. 2. Effect of SH-Chol on the formation of TBARS during copper-catalyzed oxidation of LDL. LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37°C. SH-Chol (15 μM) was added to the incubation medium at 0 h (a), 0 and 1 h after incubation (b), or 0 and 2 h after incubation (c). The values are the means ± SD of three separate experiments (triplicates for each experiment) done on the same LDL sample. *$P < 0.05$ from the value obtained by addition of SH-Chol at 0 (a), and at 0 and 2 h (c) by analysis of variance, Scheffe's test. ○, +SH-Chol; ●, –SH-Chol. See Figure 1 for abbreviations.

TABLE 1
Effect of Thiocholesterol (SH-Chol) on Copper-Catalyzed Oxidation of Low-Density Lipoprotein (LDL)[a]

Addition	TBARS (nmol MDA/mL)
None	0.65 ± 0.10
Cu^{2+}	13.25 ± 0.78
Cu^{2+} + 15 μM SH-Chol	10.19 ± 0.76
Cu^{2+} + 30 μM SH-Chol	1.05 ± 0.35[b]
Cu^{2+} + 60 μM SH-Chol	0.45 ± 0.05[b]
Cu^{2+} + 120 μM SH-Chol	0.40 ± 0.01[b]
Cu^{2+} + 30 μm SH-Chol added at the end of incubation	13.10 ± 0.52

[a]LDL (200 μg protein/mL) was incubated with 10 μM $CuSO_4$ at 37°C for 4 h in the presence or absence of SH-Chol. Thiobarbituric acid reactive substances (TBARS) contents of samples were determined as described in the Materials and Methods section. The values are the means ± SD of three separate experiments (triplicates for each experiment) done on the same LDL sample.

[b]$P < 0.001$ from the value obtained by addition of Cu^{2+} alone by analysis of variance, Scheffe's test.

DPPH, which is a typical model compound of the stable free radical, was reduced by SH-Chol, as well as by α-tocopherol and cysteine, which are typical free-radical scavengers (30). The results suggest that the antioxidant effect of SH-Chol on copper-induced oxidation of LDL may partly be due to its radical scavenging action. In addition to acting as the radical scavenger, one of the mechanisms of its inhibitory action on the initiation process of lipid peroxidation may be the formation of a Cu–SH-Chol complex as indicated by Steinrauf *et al.* (21), who have reported that the thiosterols form methylmercuric ion–thiosterol complexes.

Recently, it has been reported that the copper-induced oxidation of cholesterol present in LDL particles forms a variety

TABLE 2
Reduction of α,α-Diphenyl-β-Picrylhydrazyl[a]

Test compound	D517 nm
Tocopherol (10 μM)	0.198 ± 0.01
Tocopherol (20 μM)	0.420 ± 0.03
Cysteine (20 μM)	0.199 ± 0.01
Cysteine (40 μM)	0.415 ± 0.02
SH-Chol (20 μM)	0.187 ± 0.02
SH-Chol (40 μM)	0.397 ± 0.04

[a]The values are the means ± SD of three determinations; see Table 1 for abbreviation.

of toxic products, notably 7-OH Chol, 7-Keto Chol, 5β, 6β-epoxycholesterol, and 25-hydroxycholesterol (31). Thus, we next investigated whether SH-Chol is able to prevent the formation of oxysterols by copper-induced oxidation of LDL. As shown in Figure 3, 7-OH and 7-Keto Chol were rapidly produced by copper-induced oxidation of LDL as previously reported by us (26). However, upon addition of 30 μM SH-Chol to the incubation medium, the formation of both oxysterols was completely inhibited. At the same time, under the present experimental conditions, we have confirmed that although the amount of SH-Chol has progressively decreased the incubation, SH-Chol is not oxidized to 7-OH and 7-Keto SH-Chol (data not shown). However, we have not yet investigated other potential oxidation products of SH-Chol. In any event, the results suggest that SH-Chol can prevent the formation of oxysterols by copper-induced oxidation of LDL and that the protective action of SH-Chol on formation of oxysterols may be due to the competition between low-density lipoprotein–cholesterol and SH-Chol in this copper-induced oxidation system.

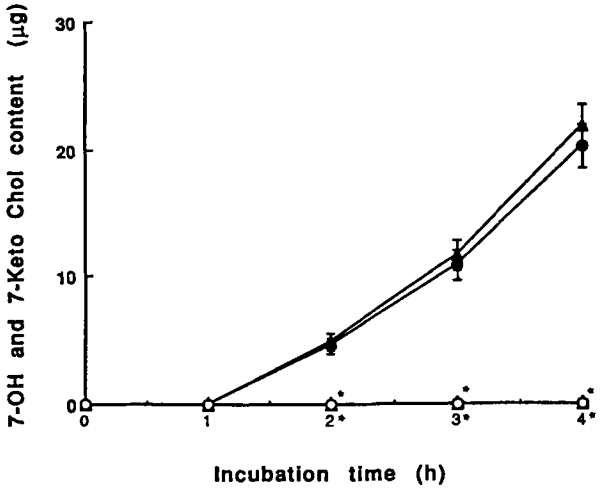


FIG. 3. Effect of thiocholesterol (SH-Chol) on 7-hydroxycholesterol (7-OH Chol) and 7-ketocholesterol (7-Keto Chol) formation during copper-catalyzed oxidation of low-density lipoprotein (LDL). LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37°C in the presence or absence of SH-Chol (30 μM). Oxysterol concentrations were determined as described in the Materials and Methods section. The values are the means ± SD of three separate experiments (triplicates for each experiment) done on the same LDL sample. *$P < 0.001$ from the value obtained by addition of Cu^{2+} alone by one-way analysis of variance, Scheffe's test. ▲, –SH-Chol (7-Keto Chol); △, +SH-Chol (7-Keto Chol); ●, -SH-Chol (7-OH Chol); O, +Sh-Chol (7-OH Chol).

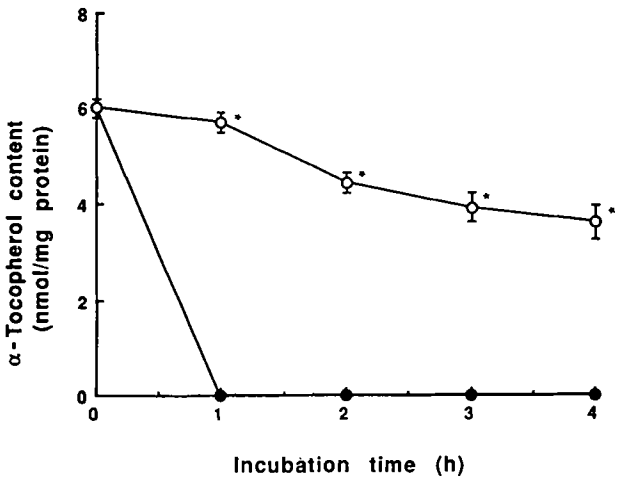


FIG. 4. Low-density lipoprotein (LDL) α-tocopherol content during copper-catalyzed oxidation of LDL in the presence or absence of thiocholesterol (SH-Chol). The incubation conditions were the same as in the experiments described in Figure 3 except for the use of 60 μM of SH-Chol. The values are the means ± SD of three separate experiments (triplicates for each experiment) done on the same LDL sample. *$P < 0.001$ from the value obtained by addition of Cu^{2+} alone by analysis of variance, Scheffe's test. O, +SH-Chol; ●, –SH-Chol.

It has been shown that the protective effects of ascorbate and probucol on LDL oxidation *in vitro* are more effective than those of α-tocopherol (32,33) and that ascorbate preserves the endogenous α-tocopherol in LDL particles during LDL oxidation *in vitro,* whereas probucol fails to preserve it (34). Therefore, we next investigated the effect of SH-Chol on the endogenous α-tocopherol content in LDL particles during copper-induced oxidation of LDL. As shown in Figure 4, when LDL was incubated with 10 μM $CuSO_4$ at 37°C for 1 h, endogenous α-tocopherol was decreased below levels of detection (<0.01 nmol/mg protein). However, addition of SH-Chol (60 μM) to the medium prior to incubation preserved approximately 60% of the initial level of α-tocopherol in LDL particles up to 4 h of incubation. Since the protective action of SH-Chol on copper-induced oxidation of LDL *in vitro* is similar to that of ascorbate (34), the protective action of SH-Chol on copper-induced oxidation of LDL likely results in the preservation of endogenous α-tocopherol in LDL particles.

The results described here suggest that SH-Chol acts as an antioxidant during the copper-induced oxidation of LDL, that the antioxidant action of SH-Chol is partly a function of its radical scavenging action, and that by reacting with lipid radicals, SH-Chol also protects α-tocopherol in LDL particles.

Experiments are in progress to study the antioxidant action and other effects of SH-Chol *in vivo.*

REFERENCES

1. Fowler, S., Shio, H., and Haley, W.J. (1979) *Lab. Invest. 41,* 372-378.

2. Schaffner, T., Taylor, K., Bartucci, E.J., Fischer-Dzoga, K., Beeson, J.H., Glagov, S., and Wissler, R.W. (1980) *Am. J. Pathol. 100*, 57–73.
3. Gerrity, R.G. (1981) *Am. J. Pathol. 103*, 181–190.
4. Brown, M.S., and Goldstein, J.L. (1983) *Annu. Rev Biochem. 52*, 223–261.
5. Steinberg, D. (1988) *Atherosclerosis Reviews 18*, 1–23.
6. Henrichsen. T., Maloney, F.M., and Steinberg, D. (1981) *Proc. Natl. Acad. Sci. USA 78*, 6499–6503.
7. Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., and Steinberg, D. (1984) *Proc. Natl. Acad. Sci. USA 81*, 3883–3887.
8. Cathcart, M.K., Morel, D.W., and Chisolm, G.M. (1985) *J. Leukoc. Biol. 38*, 341–350.
9. Heinecke, L., Rosen, H., and Chait, A. (1984) *J. Clin. Invest. 74*, 1980–1984.
10. Fong, L.G., Parthasarathy, S., Witztum, J.L., and Steinberg, D. (1987) *J. Lipid Res. 28*, 1466–1477.
11. Smith, C., Mitchinson, M.J., Aruoma, O.I., and Halliwell, B. (1992) *Biochem. J. 286*, 901–905.
12. Salonen, J.T., Salonen, K., Seppanen, K., Kantola, M., Suntioinen, S., and Korpela, H. (1991) *Br. Med. J. 302*, 756–760.
13. Yonaha, M., Ohbayashi, Y., Ichinose, T., and Sagai, M. (1982) *Chem. Pharm. Bull. 30*, 1437–1442.
14. Stacey, N.H., and Klaassen, C.D. (1981) *J. Toxicol. Environ. Health. 7*, 139–147.
15. Gabor, S., Anca, Z., and Bordas, E. (1978) *Rev. Roum. Biochim. 15*, 113–117.
16. Leedle, R.A., and Aust, S.D. (1990) *Lipids 25*, 241–245.
17. Palmanda, J.R., and Kehrer, J.P. (1993) *Lipids 28*, 427–431.
18. Mitsos, S.E., Fantone, J.C., Gallagher, K.P., Walden, K.M., Simpson, P.J., Abrams, G.D., and Schork, M.A. (1986) *J. Cardiovasc. Pharmacol. 8*, 978–988.
19. Bruno, M.K., Cohen, S.D., and Khairallah, E.A. (1988) *Biochem. Pharmacol. 37*, 4319–4325.
20. Tanaka, M., Anzai, S., Takeno, K., and Nakagawa, M. (1994) *Biol. Pharm. Bull. 17*, 1151–1154.
21. Steinrauf, L.K., Cox, B., Foster, E., Sattar, A., and Blickenstaff, R.T. (1978) *J. Pharm. Sci. 67*, 1739–1743.
22. Blickenstaff, R.T., Cox, B., Foster, E., Roberts, L., and Steinrauf, L.K. (1980) *J. Pharm. Sci. 69*, 556–558.
23. Shin, H.C., and Quinn, D.M. (1993) *Lipids 28*, 73–74.
24. Hatch, F.T., and Lees, R.S. (1968) *Adv. Lipid Res. 6*, 1–68.
25. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochem. 95*, 351–358.
26. Tanaka, M., and Kanamaru, S. (1993) *Biol. Pharm. Bull. 16*, 538–543.
27. Bieri, J.G., Tolliver, T.J., and Catignani, G.L. (1979) *Amer. J. Clin. Nutr. 32*, 2143–2149.
28. Uchiyama. M., Suzuki, Y., and Fukuzawa, K. (1968) *Yakugaku Zasshi 88*, 678–683.
29. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265–275.
30. Poirier, R.H., and Benington, F. (1952) *J. Org. Chem. 17*, 1437–1445.
31. Bhadra, S., Arshad, M.A.Q., Rymaszewski, Z., Norman, E., Wherley, R., and Subbiah, M.T.R. (1991) *Biochem. Biophys. Res. Commun. 176*, 431–440.
32. Jialal, I., Vega, G.L., and Grundy, S.M. (1990) *Atherosclerosis 82*, 185–191.
33. Steinbrecher, U.P. (1988) *Biochim. Biophys. Acta 959*, 20–30.
34. Jialal, I., and Grundy, S.M. (1991) *J. Clin. Invest. 87*, 597–601.

[Received April 29, 1994, and in final revised form January 3, 1995; Revision accepted January 16, 1995]

Thermotropic Behavior of Stratum Corneum Lipids Containing a Pseudo-Ceramide

Hiromoto Mizushima[a,*], Jun-ichi Fukasawa[b] and Toshiyuki Suzuki[b]

[a]Wakayama Research Laboratories, Kao Corporation, Wakayama 640, Japan and
[b]Tokyo Research Laboratories, Kao Corporation, Tokyo 131, Japan

ABSTRACT: The mechanism of the self-assembly of the lamellar structure of natural stratum corneum lipids (SCL) has been a subject of considerable interest. We have examined this question by using a synthetic pseudo-ceramide (sphingolipid E, SLE) which was analogous to the naturally occurring ceramide type 2. The thermotropic properties and the structural characteristics of SLE, together with other main components of SCL, fatty acids, and cholesterol, were investigated by differential scanning calorimetry and X-ray analysis. A mixture of SLE and stearic acid was in a stable α-form having a lamellar structure, which is very similar to that of natural SCL. However, lipid mixtures in which stearic acid were replaced by oleic acid did not form lamellar structures, and existed in the crystalline states. This indicates that the stable bilayer formation of the natural SCL is strongly dependent on the molecular fatty acid structure. Moreover, incorporation of cholesterol (0–50%) into equimolar mixtures of SLE/stearic acid and of SLE/oleic acid caused a marked decrease of melting entropies, while the aggregation states of both systems were not changed. This effect of cholesterol can be attributed to the disorder of the molecular packing. These results suggest that the hydrophobic interactions between the SCL are important for bilayer formation as are the hydrophilic interactions between the polar groups.
Lipids 30, 327–332 (1995)

The stratum corneum lipids (SCL) which exist in the intercellular space of the stratum corneum are known to provide an epidermal barrier against water loss as well as protection against environmental, chemical, and biological stress (1–3). It is established now that the SCL, which consist of ceramides, fatty acids, cholesterol, and cholesteryl esters, retain water in the stratum corneum by forming lamellar structures (4,5). Elias (3,6) suggested that the presence of ceramides and sphingolipids is the basis for the structural organization of SCL in bilayers. Wertz and Downing (7) determined the structures and the composition of the ceramides. Wertz *et al.* (8) showed that ceramides could take part in the bilayer formation, and that fatty acids were essential for this to occur (9). Imokawa and co-workers (4,10–12) reported that ceramides play an important role in the water-retaining properties of stratum corneum. Furthermore, they found that a synthetic pseudo-ceramide, named sphingolipid E (SLE), which had a molecular structure analogous to that of the naturally-occurring ceramide type 2 (7), exhibited water-retaining properties similar to those of the natural lipids (12).

The dermatological properties of the SCL and their interactions with ceramides are under active investigation (13–18). Although the ceramides have apparent importance in the structure and function of SCL, relatively little effort has been directed toward elucidating their structure–function relationships. Studying the physicochemical properties of SLE in a model SCL mixture would greatly facilitate our understanding of the behavior of natural ceramides.

Our preliminary study (19) of the thermotropic phase behavior of the pseudo-ceramide SLE suggested that SLE showed polymorphism, as was reported for natural SCL (9,13,17,20). Moreover, we examined the aggregation structures of the equimolar mixtures of SLE/cholesterol; SLE/stearic acid; and SLE/cholesteryl isostearate (a cholesteryl ester of methyl-branched octadecanoic acid), and we found that only the equimolar mixture of SLE and stearic acid formed a stable lamellar structure (19,21). In addition, the equimolar mixture of SLE and cholesterol was in liquid-crystalline state when it was prepared, whereas the liquid-crystalline state was unstable and changed to a rigid-crystalline state in a few days. These results suggest that the interaction between ceramides and fatty acids is important for the bilayer formation of natural SCL. The effect of cholesterol on the thermotropic behavior of SLE is also a subject of considerable interest.

In the present study, we report the detailed thermotropic behavior of an SLE/stearic acid system to clarify the role of fatty acid in the self-assembly of natural SCL. Furthermore, we focus on the hydrophobic interactions between these lipids. The thermotropic properties and the structural characteristics of SLE in mixtures of stearic acid, oleic acid, and cholesterol were investigated using differential scanning calorimetry (DSC) and X-ray diffraction.

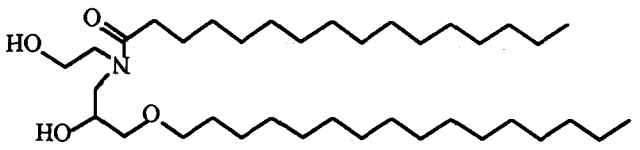
*To whom correspondence should be addressed at Wakayama Research Laboratories, Kao Corporation, 1334 Minato, Wakayama 640, Japan.

Abbreviations: ΔHm, melting enthalpy; ΔSm, melting entropy; Tm, melting point; DSC, differential scanning calorimetry; SCL, stratum corneum lipids; SLE, sphingolipid E.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Materials. The pseudo-ceramide *N*-(3-hexadecyloxy-2-hydroxypropyl)-*N*-2-hydroxyethyl hexadecanamide, SLE, was obtained from Kao Co. (Tokyo, Japan), and the purity was greater than 99% as measured by high-performance liquid chromatographic analysis (Scheme 1). Stearic acid, oleic acid, and cholesterol of reagent-grade were purchased from Wako Pure Chemical Industry (Osaka, Japan). The compounds were dried *in vacuo* and were used without further purification. The water content of all lipids was checked by Karl Fischer's method and were found to be less than 0.1%.



SCHEME 1

Preparation of samples. The lipids containing SLE (5–10 g) were weighed into glass test tubes with a Teflon-sealed screw cap and were melted at 90–100°C to ensure homogeneous mixing. Then, the samples were cooled to 25°C and maintained at that temperature for seven days or more, after which the DSC and X-ray measurements were started.

DSC. DSC analyses were carried out on a DSC-100 instrument (Seiko Instrument & Electronics Ltd., Tokyo, Japan). The lipid mixture (10–20 mg) was put into a silver DSC pan (Seiko). The pan was sealed and placed in the DSC cell along with a vacant reference pan. Then the sample was heated from 20 to 100°C at 2°C/min. The melting point (T_m) was determined by extrapolation to the baseline of the most rapid rise in the excess heat capacity curve as a function of temperature. The melting enthalpy (ΔH_m) was determined by integrating the area under the curve from a plot of the excess heat capacity as a function of temperature. The melting entropy (ΔS_m) was calculated from Equation 1:

$$\Delta S_m = \Delta H_m / T_m \quad [1]$$

X-Ray diffraction. The aggregation structure of the lipids was identified by powder X-ray diffraction with a wide-angle diffractometer, XD-7A (Shimadzu Co., Kyoto, Japan). Cu-Kα radiation ($\lambda = 1.54$ Å) was used in X-ray diffractometries. The lipid mixtures were placed on the X-ray diffraction glass plate in a sample holder, and then studied at 25°C.

RESULTS

Interaction of SLE and fatty acids. In Figure 1, representative DSC curves of SLE/stearic acid mixtures are given. On the basis of calorimetric scans, it is clear that stearic acid at concentrations as low as 30% has a marked effect on the thermotropic behavior of SLE (Fig. 1, curves A–F). As the stearic

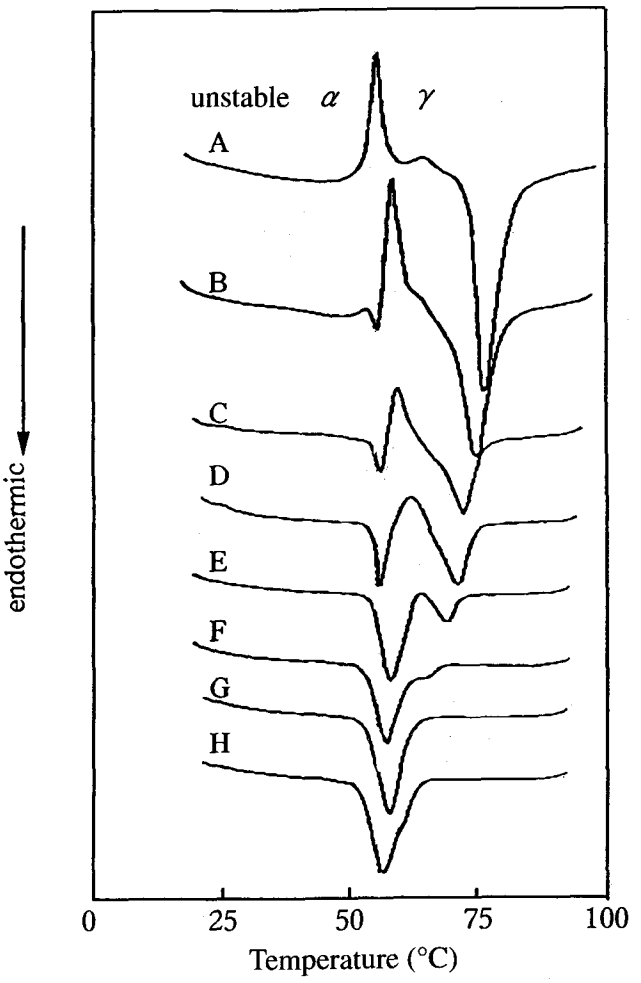


FIG. 1. Differential scanning calorimetry curves of sphingolipid E/stearic acid mixtures with weight fraction of stearic acid being: (A) 0, (B) 0.05, (C) 0.10, (D) 0.15, (E) 0.20, (F) 0.30, (G) 0.40, and (H) 0.50.

acid content was increased, the endothermic peak at around 75°C (γ-crystal to liquid) decreased and shifted to a lower temperature (Fig. 1, curves A–F), and then disappeared when the weight fraction of stearic acid was 0.4 or more (Fig. 1, curves G and H).

The exothermic peak of pure SLE at around 50°C, which may be attributed to a close packing of hydrocarbon chains of SLE molecules (19), was decreased as the stearic acid content increased. Concurrently, a new endothermic peak at around 50°C appeared. The DSC curves of the SLE/stearic acid mixture containing 40% stearic acid showed only one broad endothermic transition at around 50°C (Fig. 1, curve G). Further, this mixture showed a strong single peak at 4.1 Å in the X-ray diffraction (Fig. 2), indicating that the lipid mixture was in the α-form at 25°C.

The T_ms and the ΔH_ms of the SLE/stearic acid system were plotted against mole fraction of stearic acid (Fig. 3). Figure 3 clearly shows that the aggregation of SLE and stearic acid changes the state of SLE from the γ-form to the α-form. To clarify the effect of unsaturation between SLE and fatty acid, the thermotropic behavior and the aggregation structure of the

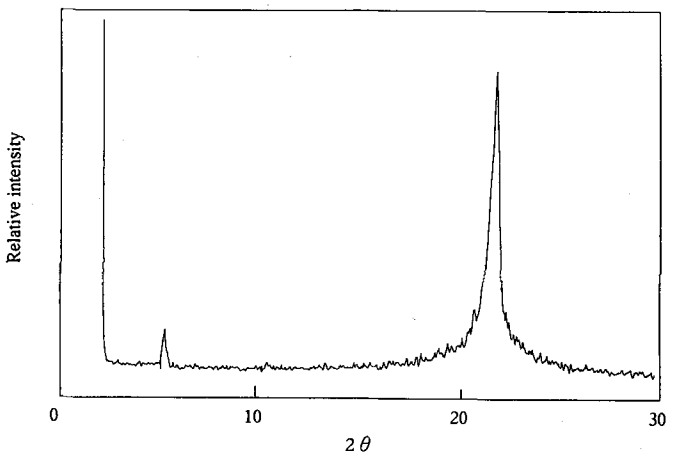


FIG. 2. X-Ray diffraction pattern at 25°C of sphingolipid E/stearic acid mixture with weight fraction of stearic acid being 0.40.

SLE/oleic acid system were examined. The DSC curves and the X-ray diffraction patterns are shown in Figure 4 and Figure 5, respectively.

As shown in Figure 4, all DSC curves of the SLE/oleic acid mixtures (weight fraction of oleic acid = 0.1–0.5) showed a single endothermic peak at higher temperatures than the melting points of SLE/stearic acid mixtures. Figure 6 clearly shows the states of the mixtures to be γ-crystal. In this system, the temperature of phase transition (γ-crystal to liquid) was shifted to the lower temperature, and the ΔH_m (Fig. 6) decreased when the oleic acid content was increased. From the X-ray diffraction patterns, the stable lamellar structure was not observed in the SLE/oleic acid mixtures containing 10–50% oleic acid.

Interaction of cholesterol and equimolar mixture of SLE and fatty acids. Changes in the T_m and ΔH_m of SLE/fatty acid (1:1 mole ratio)/cholesterol system were shown in Table 1, and the calculated ΔS_ms were plotted as a function of cholesterol content in Figure 7. Incorporation of cholesterol into the equimolar mixtures of SLE/stearic acid and SLE/oleic acid caused a marked decrease of the phase transition enthalpy and the ΔS_m. It can be seen that the extent of the entropy decrease of the SLE/stearic acid/cholesterol system was larger than that of the SLE/oleic acid/cholesterol system in the range of 22–53 mol% (20–50 wt%) cholesterol (Fig. 7 and Table 1). On the other hand, based on the X-ray data, the incorporation of cholesterol did not change the aggregation states of either system when the mole fraction of cholesterol was 53 mol% (50 wt%) or less. The typical X-ray patterns of the SLE/fatty acid/cholesterol mixtures with 20 wt% of cholesterol (SLE/fatty acid/cholesterol = 54:26:20 weight ratio) showed that the stearic acid mixture was in α-form and the oleic acid mixture in γ-form (Fig. 8).

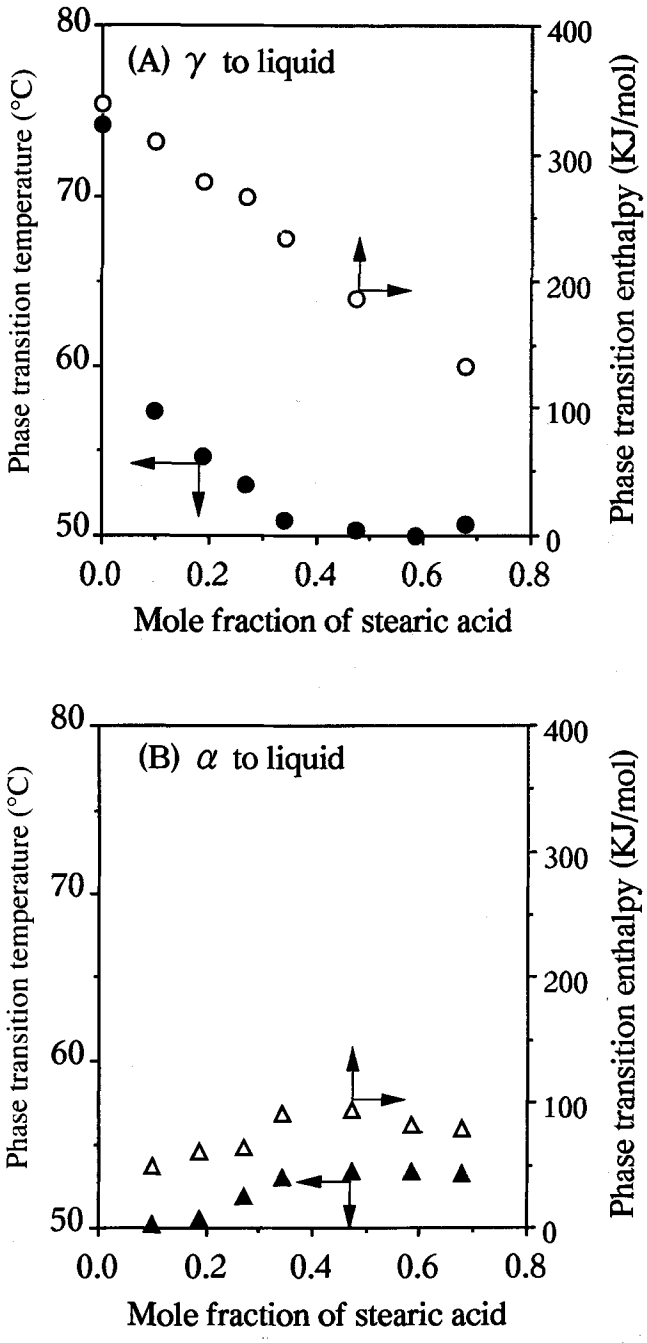


FIG. 3. Phase transition temperature (○, △) and phase transition enthalpy (●, ▲) of sphingolipid E/stearic acid system. A indicates the phase transition from γ to liquid and B, the phase transition from α to liquid.

DISCUSSION

Pure SLE exists as the monoclinic γ-crystal (22–26) in which the methylene chains are packed in a parallel fashion. In the γ-form, four strong diffraction peaks (4.24, 4.13, 3.78, and 3.62 Å) are observed in X-ray diffraction in the short-spacing region (19). The γ-crystal of SLE undergoes an endothermic transition at around 75°C to form an isotropic liquid. Upon cooling, the melted SLE undergoes a state change from a supercooled liquid to a lamellar α-form at around 50°C. In the α-form, a strong single peak is observed (19,20). The characteristic X-ray diffraction pattern of the α-form indicates that the alkyl chains are hexagonally packed and rotating within

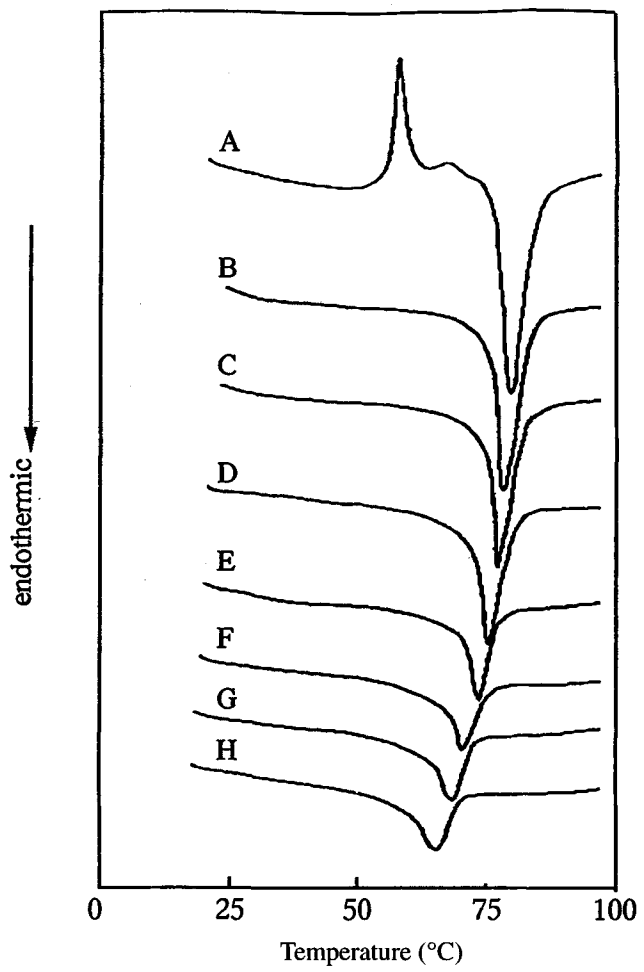


FIG. 4. Differential scanning calorimetry curves of sphingolipid E/oleic acid mixtures with weight fraction of oleic acid being: (A) 0, (B) 0.05, (C) 0.10, (D) 0.15, (E) 0.20, (F) 0.30, (G) 0.40, and (H) 0.50.

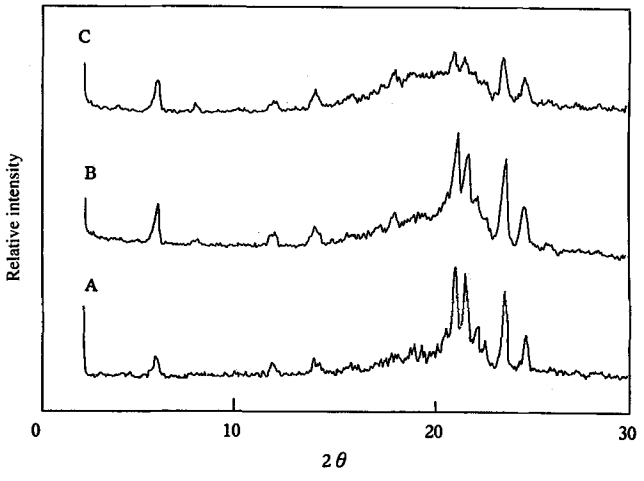


FIG. 5. X-Ray diffraction patterns at 25°C of sphingolipid E/oleic acid mixtures with weight fraction of oleic acid being: (A) 0.30, (B) 0.40, and (C) 0.50.

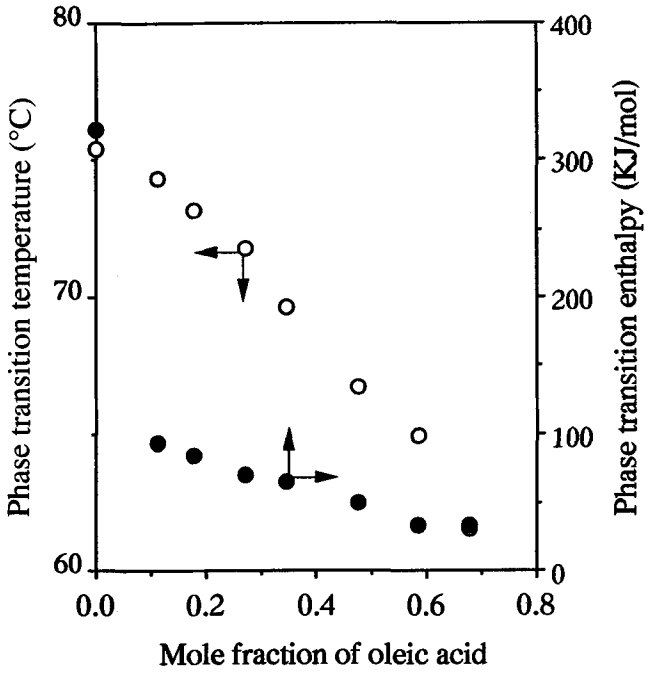


FIG. 6. Phase transition temperature (○) and phase transition entropy (●) of sphingolipid E/oleic acid system.

lamellar planes (27,28). However, the α-form of SLE is metastable and changes to the monoclinic γ-crystal upon long-term storage (19). The results from the present study show that fatty acids having long hydrocarbon chains affect the thermotropic properties (Figs. 1 and 4) and the aggregation structure (Figs. 2 and 5) of the synthetic pseudo-ceramide. The thermotropic behavior is similar to that observed in the glucosyl-ceramide and phospholipid system (20). The difference in the thermotropic behavior and the stable states of the SLE/stearic acid system and of the SLE/oleic acid system clearly shows that hydrophobic interactions between the lipid molecules are as important for determining the aggregation state as are the hydrophilic interactions (9,13,19,20).

Investigation of the monolayer behavior at the air–water interface of ceramides having various hydrophobic moieties showed that the aggregation structure of the condensed state

TABLE 1
Melting Point (T_m) and Melting Enthalpy (ΔH_m) of SLE/Fatty Acid/Cholesterol System[a]

	Fatty acid			
	Stearic acid[b]		Oleic acid[c]	
Cholesterol (wt%)	T_m(°C)	ΔH_m(KJ/mol)	T_m(°C)	ΔH_m(KJ/mol)
0	58.2	43.1	67.3	43.1
10	54.4	38.8	64.7	39.3
20	54.6	23.9	62.3	31.8
30	54.2	11.0	63.3	27.0
40	59.3	6.2	63.5	20.6
50	57.1	7.4	63.5	18.3

[a]SLE/fatty acid = 1:1 mole ratio.
[b]α→Liquid, [c]γ→Liquid.

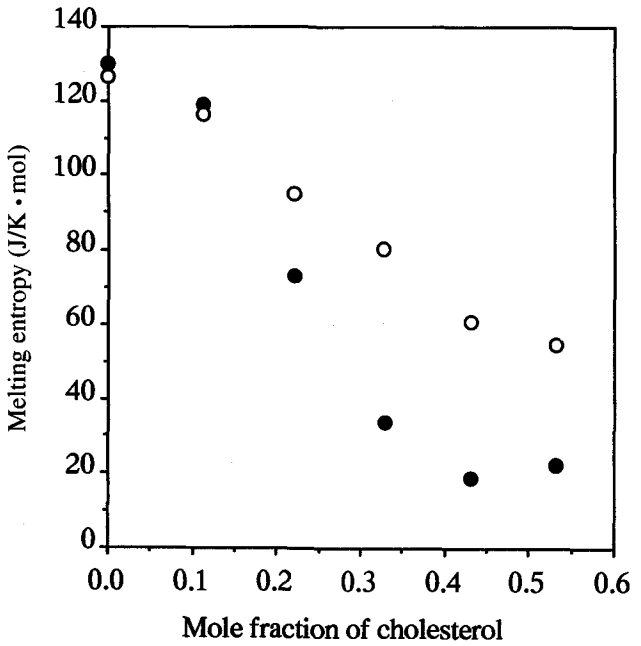


FIG. 7. Melting entropy of sphingolipid E (SLE)/fatty acid/cholesterol system (SLE/fatty acid = 1:1 mole): (●), the melting entropy of SLE/stearic acid/cholesterol system in α-form; (○), the melting entropy of SLE/oleic acid/cholesterol system in γ-form.

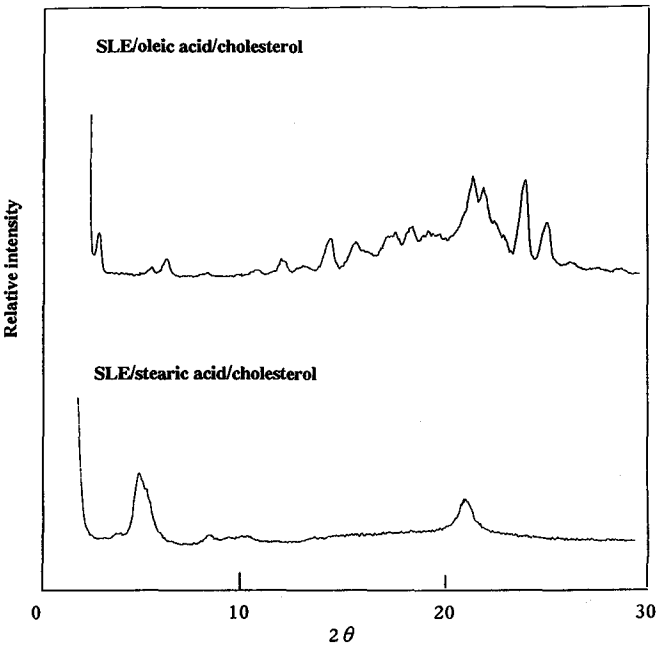


FIG. 8. X-ray diffraction patterns at 25°C of sphingolipid E (SLE)/fatty acid/cholesterol mixtures with weight fraction of cholesterol being 0.2 (SLE/fatty acid = 1:1 mole).

is dependent on the nature of the hydrophobic part of the molecules, and that the *cis* double bond in the fatty acid reduces the packing to limit the lateral interactions of polar groups (18). In a study of the thermotropic behavior of glucosyl-ceramide and phospholipids, it was found that the intermolecular interaction among the glycosphingolipid and phospholipid molecules is strongly dependent on the acyl chain of the phospholipid (20). Our present data are consistent with these observations. Therefore, changes in the structure of the hydrophobic part of fatty acid would be anticipated to affect the molecular packing and/or the mobility of the pseudo-ceramide and fatty acid mixture. In the α-form, the interaction between lipids is relatively small, since the mobility of the lipid molecule in the α-form is larger than that in the crystalline state (27,28). For the formation of the stable α-form, it is believed that strong hydrophilic interaction; such as hydrogen bonding between the hydrophilic portions of the lipids, would be necessary. Since oleic acid has a *cis* double bond at the center of the molecule, close packing of oleic acid with an SLE molecule bearing two straight hydrocarbon chains would be less likely than stearic acid. The decrease in the molecular packing should limit the lateral interaction of the polar group (18). Therefore, oleic acid cannot be accommodated in the α-form, but forms the γ-crystalline with SLE molecule.

On the other hand, it was observed that the incorporation of cholesterol into SLE/fatty acid system decreased the melting entropy of the lipids (Fig. 7) without any phase transition (Figs. 2, 6, and 8). The lipid molecules change their aggregation state from the α-form and/or γ-crystalline to the isotropic liquid by melting. Therefore, the magnitude of the ΔS_m associated with the T_m should reflect the degree of disorder of the molecular packing. Thus, the decrease of the ΔS_m in the SLE/stearic acid/cholesterol system and in the SLE/oleic acid/cholesterol system (Table 1 and Fig. 7) by the incorporation of cholesterol can be attributed to the disorder in molecular packing. Cholesterol, a major component of almost all biological membranes, seems to generally play an architectural role, reducing the flexibility of the hydrocarbon chains of lipids and stiffening the membranes. This stiffening effect, well-known as the cholesterolic effect, is accompanied by a reduction in the surface area occupied by the lipid mixture and by a decrease in permeability (29). Additionally, cholesterol broadens or eliminates the gel-to-liquid-crystalline phase transition of phospholipids containing straight acyl chains (29), but this mechanism is not clear. Wiedmann and Salmon (13) examined the effect of cholesterol on the thermotropic phase transition of the hydrated natural hydroxyceramides. They reported that cholesterol caused a broadening of the phase transition at relatively low levels as well as a decrease in the peak transition temperature. In the SLE/stearic acid system and in the SLE/oleic acid system, the incorporation of cholesterol enhanced the mobility of the lipids and decreased the ΔS_m. This behavior is close to that of the several natural systems mentioned above. Moreover, the extent of the decrease of ΔS_m in the SLE/stearic acid/cholesterol system was larger than that in the SLE/oleic acid/cholesterol system when cholesterol content was 20–50%. We are not able to explain this phase behavior at the present time.

In conclusion, this study showed that an anhydrous mixture of the pseudo-ceramide and stearic acid formed a stable lamellar structure, and that the difference of the hydrocarbon

chain of fatty acid affected the molecular packing of the artificial SCL. Moreover, it was noted that incorporation of cholesterol into the pseudo-ceramide/fatty acid system gave rise to a marked decrease of ΔS_m without any phase transition. Further studies on the phase behavior and dynamic aspects of the artificial SCL in an aqueous system are needed to explain the effects of cholesterol and fatty acid unsaturation that we have discussed.

REFERENCES

1. Elias, P.M. (1981) *Arch. Dermatol. Res. 270*, 95–117.
2. Lampe, M.A., Burlingame, A.L., Whitney, J., Williams, M.I., Brown, B.E., Roitman, E., and Elias, P.M. (1983) *J. Lipid Res. 24*, 120–130.
3. Elias, P.M. (1983) *J. Invest. Dermatol. 80*, 44–49.
4. Imokawa, G., Akasaki, S., Hattori, H., and Yoshizuka, N. (1986) *J. Invest. Dermatol. 87*, 758–761.
5. Grubauer, G., Freingold, K.R., Harris, R.M., and Elias, P.M. (1989) *J. Lipid Res. 30*, 89–96.
6. Elias, P.M. (1981) *Int. J. Dermatology 20*, 1–19.
7. Wertz, P.W., and Downing, D.T. (1983) *J. Lipid Res. 24*, 759–765.
8. Wertz, P.W., Miethke, M.C., Long, S.A., Strauss, J.S., and Downing, D.T. (1985) *J. Invest. Dermatol. 84*, 410–412.
9. Wertz, P.W., Abraham, W., Landmann, L., and Downing, D.T. (1986) *J. Invest. Dermatol. 87*, 582–584.
10. Imokawa, G., Kuno, H., and Kawai, M. (1991) *J. Invest. Dermatol. 96*, 845–851.
11. Imokawa, G., and Hattori, M. (1985) *J. Invest. Dermatol. 84*, 282–284.
12. Imokawa, G., Akasaki, S., Kawamata, A., Yano, S., and Takaishi, N. (1989) *J. Soc. Cosmet. Chem. 40*, 273–285.
13. Wiedmann, T.S., and Salmon, A. (1991) *Lipids 26*, 364–368.
14. Friberg, S.E., and Osborne, D.W. (1985) *J. Disp. Sci. and Techn. 6*, 485–495.
15. Bouwstra, A., Gooris, G.S., van der Spek, J.A., and Bra, W. (1985) *Progr. Colloid Polym. Sci. 88*, 110–122.
16. Rehfeld, S.J., Plachy, W.Z., Williams, M.L., and Elias, P.M. (1988) *J. Invest. Dermatol. 91*, 499–505.
17. Abraham, W., and Downing, D.T. (1991) *Biochim. Biophys. Acta 1068*, 189–194.
18. Löfgren, H., and Pasher, I. (1977) *Chem. Phys. Lipids 20*, 273–284.
19. Suzuki, T., Fukasawa, J., Iwai, H., Yamashita, O., and Sugai, I. (1992) *IFSCC 17th International Congress Yokohama*, Vol. 1, 3–28.
20. Mizushima, H., Fukasawa, J., and Suzuki, T. (1994) *J. Jpn. Oil Chem. Soc. (YUKAGAKU) 43*, 656–658.
21. Barenholz, Y., Bach, D., Thompson, T.V., Freire, E., Correa-Freire, M., and Miller, I.R. (1989) *Thermochimica Acta 148*, 355–363.
22. Suzuki, M., and Ogaki, T. (1985) *J. Am. Oil Chem. Soc. 62*, 1600–1604.
23. Kobayashi, M., Kaneko, F., Sato, K., and Suzuki, M. (1986) *J. Phys. Chem. 90*, 6371–6378.
24. Hiramatsu, N., Sato, T., Inoue, T., Suzuki, M., and Sato, K. (1990) *Chem. Phys. Lipids 56*, 59–63.
25. Tanaka, K., Seto, T., and Hayashida, T. (1957) *Bull. Inst. Chem. Res. Kyoto Univ. 35*, 123–139.
26. Tanaka, K., Seto, T., Watanabe, A., and Hayashida, T. (1959) *Bull. Inst. Chem. Res. Kyoto Univ. 37*, 281–293.
27. Larsson, K. (1967) *Zeit. Phys. Chem. 56*, 173–198.
28. Larsson, K. (1976) *Food Emulsions* (Friberg, S.E., ed.), Marcel Dekker, New York, pp. 39–66.
29. Demel, R.A., and De Kruijff, B. (1976) *Biochim. Biophys. Acta 457*, 109–132.

[Received July 12, 1993, and in revised form February 11, 1995; Revision accepted February 22, 1995]

Monohexosylceramides of Larval and Adult Forms of the Tapeworm, *Spirometra erinacei*

Yasushi Kawakami[a,b], Hisako Kojima[b], Kazuo Nakamura[b], Minoru Suzuki[c], Akihiko Uchida[a], Yoshihiko Murata[a] and Yoichi Tamai[b,*]

[a]Department of Environmental Biology, Azabu University, Sagamihara, Kanagawa 229, [b]Department of Biochemistry, Kitasato University School of Medicine, Sagamihara, Kanagawa 228, and [c]Department of Membrane Biochemistry, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT: The occurrence of glycosphingolipids with unique carbohydrate structures in different species of cestode, Platyhelminth, which had been shown previously, prompted us to study the molecular species of the monohexosylceramides (cerebrosides) in the pseudophyllidean cestode, *Spirometra erinacei*. The purpose of the study was to obtain a basis for future investigations of the physiological role of glycolipids in parasitism. Cerebrosides were isolated from *S. erinacei* at two growth stages, i.e., from the larval form (plerocercoid) and from the adult tapeworms (intestinal form). The cerebrosides were separated into four subfractions by silica gel column chromatography, and their constituents were analyzed by gas–liquid chromatography, gas chromatography/mass spectrometry, and high-performance liquid chromatography/mass spectrometry. The hexoses of the cerebrosides consisted primarily of galactose in both growth stages, while only a small amount of glucose was detected. The ceramides were composed of sphinganine (d18:0) and phytosphingosine (t18:0) as sphingoid bases, and of nonhydroxy fatty acids ranging from C_{16} to C_{30} and hydroxy stearic acid (18h:0). The cerebrosides of adult tapeworms contained more 18h:0 than those of plerocercoids. The combination of hexoses and ceramides in the cerebroside molecules was slightly different in the two growth stages: the glucocerebrosides of plerocercoids contained only d18:0-nonhydroxy fatty acids in their ceramide moieties, whereas those of adult tapeworms contained varying ceramide moieties. Our data indicate that the molecular species of glycolipids present were essentially homeostatic throughout growth in spite of the entirely different environmental conditions, although there were slight differences in the hexose distribution in the two growth stages. *Lipids 30,* 333–337 (1995).

Glycosphingolipids are located on the outer surface of the plasma membrane of most living organisms and are known to participate in various important events occurring on the cell surface (1,2). In parasites, glycosphingolipids may also be involved in processes essential for parasite survival, such as mediation of the host-parasite interaction and in host immune response. Recent structural analyses of parasitic helminth glycosphingolipids have revealed some species-characteristic glycolipid differences and differences in immunological antigenicity (for reviews, see Refs. 3 and 4). The findings point toward a possible biological role of glycosphingolipids in parasitism.

We recently isolated a fucosylated glycosphingolipid (SEGLx) with a novel carbohydrate structure from plerocercoids of *Spirometra erinacei* (5). This prompted us to study in more detail the distribution of glycosphingolipids in this parasite. In the present study, we determined the molecular species of the cerebrosides in two growth stages of *S. erinacei*, i.e., in the larval (plerocercoids) and in the adult forms. Although the compositional heterogeneity of cerebrosides in different cestode species has been reported (6–11), the cerebrosides of *S. erinacei* have not been characterized so far. Based on the present findings, we will discuss differences in cerebroside compositions at different growth stages, species differences, and their relationship with putative precursors of SEGLx.

MATERIALS AND METHODS

Collection of plerocercoids and adult worms. Plerocercoids of *S. erinacei* were collected from snakes, *Elaphe quadrivirgata*, in Japan. The plerocercoids were quickly removed from the muscle and subcutaneous tissues of snakes, and washed thoroughly with 0.8% NaCl. Adult tapeworms were obtained from cats infected with plerocercoids for 20 d. Tapeworms were collected from the cat intestines and washed with 0.8% NaCl. Totals of approximately 200 g of plerocercoids and 150 g of adult tapeworms were used in the present investigation.

Extraction and purification of glycolipids. Plerocercoids and tapeworms were extracted with 20 vol of chloroform/methanol (2:1, vol/vol), followed by 10 vol of chloroform/methanol/water (1.0:1.0:0.1, by vol). The filtrates were combined and concentrated to dryness in a rotary evaporator. Following removal of nonlipid contaminants by repetitive ex-

*To whom correspondence should be addressed at Department of Biochemistry, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan.

Abbreviations: FAB–MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography/mass spectrometry; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl.

Copyright © 1995 by AOCS Press

traction with chloroform/methanol (2:1, vol/vol), the material soluble in this solvent (total lipids) amounted to 4.0 g from plerocercoids and 2.3 g from adult cestodes.

Total lipids were subsequently chromatographed on a column of DEAE-Toyopearl (Tosoh Co., Tokyo, Japan), acetate form, and neutral glycolipids were separated from acidic lipids as described (12). Neutral glycolipids were further fractionated by Iatrobeads (6RS-8060 1 × 90 cm; Iatron Laboratories, Tokyo, Japan) column chromatography, using a linear gradient solvent system, comprising 5 column vol of chloroform/methanol (98:2, vol/vol) to chloroform/methanol/water (60:40:5, by vol), at the flow rate of 2 mL/min. The resultant cerebroside fraction was purified by high-performance liquid chromatography (HPLC) (Model CCPS; Tosoh Co.) on a column of ODS 80TM (4.6 × 150 mm; Tosoh Co.). It was confirmed that the isolated cerebrosides did not contain any other glycolipids on thin-layer chromatography (TLC).

Analytical methods. Purified glycolipids were methanolyzed with 5% methanolic HCl at 100°C for 3 h. Fatty acid methyl esters were extracted from the methanolysate using *n*-hexane. The resultant methanolic phases were dried under a stream of nitrogen and the residue trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane (10:2:1, by vol). The trimethylsilyl (TMS) derivatives of carbohydrates were subsequently analyzed by gas–liquid chromatography (GLC) on a SPB-1 fused-silica capillary column (0.32 mm × 30 m; Supelco Inc., Bellefonte, PA). The column temperature was programmed from 60 to 190°C at the rate of 20°C/min, held at 190°C for 1 min, and then elevated to 260°C at the rate of 10°C/min.

Fatty acid methyl esters were analyzed by GLC on the same column as above. The column temperature was programmed from 50 to 200°C at the rate of 20°C/min, and then elevated to 300°C at the rate of 10°C/min. For determination of sphingoid bases of glycolipids, the material was hydrolyzed with aqueous methanolic HCl at 85°C for 3 h (13). After removal of fatty acid methyl esters, sphingoid bases in the methanolic phase were extracted using diethyl ether under strongly alkaline conditions, and then analyzed as TMS derivatives by GLC, as described above. The column temperature was programmed from 50 to 240°C at the rate of 20°C/min, and then raised to 260°C at the rate of 2°C/min.

Gas chromatography/mass spectrometry (GC/MS) was performed with a Shimadzu QP-1100EX apparatus (Shimadzu, Kyoto, Japan) on a HiCap CBP1 fused-silica capillary column (0.33 mm × 25 m; Shimadzu), with He as the carrier gas, which was applied at the flow rate of 1 mL/min. MS was carried out at an electron energy of 70 eV and an ion source temperature of 250°C.

Fast atom bombardment mass spectrometry (FAB–MS). Negative-ion FAB–MS of cerebrosides was performed using a JMS-HX110 double focusing mass spectrometer equipped with an FAB ion source and a JMA-DA5000 data system (JEOL, Tokyo, Japan), with triethanolamine as the matrix (14). The accelerating voltage was set at 8.0 kV and the primary beam for bombardment at 6.0 kV Xe^0.

Micro HPLC/FAB–MS analysis. Micro HPLC/FAB–MS was performed using a newly developed system, as described previously (14). Briefly, cerebrosides isolated from *S. erinacei* were injected onto a column (Develosil ODS-5, 0.3 mm × 150 mm; Nomura Chemical Co., Aichi, Japan) and separated by elution with 10% *n*-hexane in methanol containing 0.9% glycerol, which was used as the matrix, at the flow rate of 6 μL/min. Liquid nitrogen was introduced into the trap of the oil diffusion pump in order to keep the vacuum as high as possible. Mass chromatography was performed for the detection of the ions from *m/z* 300 to 1,500 at intervals of 5 s.

RESULTS AND DISCUSSION

Composition of glycosphingolipids. A total of 48 mg of cerebrosides was extracted from 200 g of plerocercoids, while 150 g of adult tapeworms provided 24 mg of cerebrosides. Figure 1 shows that cerebrosides from both plerocercoids and adult worms were separated into four fractions, represented by single bands, on TLC. The relative contents of L1, L2, L3, and L4, which were determined by TLC–densitometry, were 1:4:1:0.3 (plerocercoids), while those of A1, A2, A3, and A4 were 1:9:17:3 (adult tapeworms). The results show that the third fraction (A3), followed by the fourth fraction (A4), increased most conspicuously with growth.

GLC analysis of the hexose composition of the cerebrosides showed that L1 consisted of galactose (69%) and glu-

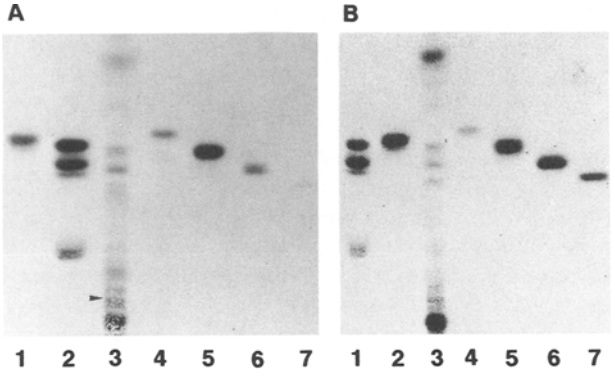


FIG. 1. Thin-layer chromatography of isolated cerebrosides of *Spirometra erinacei*. A. Plerocercoids. Lane 1, authentic glucosylceramide from human Gaucher's spleen; lane 2, authentic galactosylceramide and sulfatide from bovine brain; lane 3, total glycolipids of plerocercoids; lanes 4, 5, 6, and 7, purified cerebroside subfractions L1, L2, L3, and L4, respectively. B. Adult tapeworms. Lane 1, galactosylceramide and sulfatide from bovine brain; lane 2, glucosylceramide from human Gaucher's spleen; lane 3, total glycolipids of adult tapeworms; lanes 4, 5, 6, and 7, purified cerebroside subfractions A1, A2, A3, and A4, respectively. The arrowhead indicates SEGLx, a recently discovered glycosphingolipid (Ref. 5). Glycolipids were developed on a silica gel HPTLC plate (Merck, Darmstadt, Germany) with chloroform/methanol/water (65:25:4, by vol), and visualized by spraying with orcinol/H_2SO_4 reagent followed by heating.

cose (31%), whereas L2–L4 contained galactose only (Table 1). The subfractions of the cerebrosides from adult tapeworms (A1–A4) contained small amounts of glucose (range, 7–13%) in addition to galactose (range, 87–93%) (Table 2). Thus, the distributions of glucose and galactose in the four subfractions were found to be slightly different between plerocercoids and adult tapeworms. In previous studies, cerebrosides obtained from *S. mansonoides* tegument (6) and *Taenia crassiceps* metacestodes (7) were found to contain galactose as the only sugar component, while those of *Echinococcus multilocularis* metacestodes (8) were comprised mainly of galactose, with glucose constituting 9% of the cerebroside hexose.

Sphingoid bases consisted of two components, sphinganine (d18:0) and phytosphingosine (t18:0). Compositional analysis revealed that the distribution of the two components was almost identical in plerocercoids and the adult form (Tables 1 and 2): L1 and A1 consisted of only d18:0, while L4 and A4 contained large amounts of t18:0 (79 and 67%, respectively). Based on these results and those of previous studies indicating that galactosylceramides of *S. mansonoides* contained d18:0 and t18:0 but not d18:1 (6), we suspect that cerebrosides from *Spirometra* species contain only saturated sphingoid bases. This is in contrast to cerebrosides from *E. multilocularis* (8), *T. crassiceps* (7), and *Metroliasthes coturnix* (11), which contain detectable amounts of d18:1.

The fatty acid composition of *S. erinacei* cerebrosides was comprised of nonhydroxy fatty acids, ranging from C_{16} to C_{30}, and hydroxy stearic acid (18h:0) (Tables 1 and 2). Plerocercoid glycolipids L1 and L2 and adult tapeworm glycolipids A1 and A2 contained only nonhydroxy fatty acids, including components longer than C_{26}. On the other hand, plerocercoid L3 and adult tapeworm A3 contained both nonhydroxy and hydroxy fatty acids, nonhydroxy components consisting of short-chain components such as C_{16} and C_{18}, while 18h:0 amounted to nearly half the total. Plerocercoid L4 and adult tapeworm A4 contained mainly 18h:0, together with 18:0 as the other major species present. Although the overall distribution of cerebroside fatty acids was similar in plerocercoids and adult tapeworms, the abundance of long-chain monounsaturated components, such as 28:1 and 30:1 in plerocercoids, and that of the hydroxy component (18h:0) in adult forms, were notable. The results of previous studies indicated that cerebrosides from *E. multilocularis* (8), *M. coturnix* (11), and *T. crassiceps* (7) contain straight-chain fatty acids that are longer than those of *S. erinacei* found in the present study. The composition of hydroxy fatty acids, however, differed with the species. For example, *E. multilocularis* and *M. coturnix* cerebrosides contained long-chain 2-hydroxy components such as C_{24}, C_{26}, and C_{28} (8,11), whereas the glycolipid in *T. crassiceps* lacked hydroxy components (7).

Ceramide species of cerebrosides. To examine the combination of sphingoid bases and fatty acids composing ceramide moieties, the whole molecules of cerebroside subfractions were analyzed by MS. Negative-ion FAB–MS spectra of glycolipids showed major pseudo-molecular ions ($[M - H]^-$) at *m/z* 728, 840, and 866 (L1 and A1), 700, 728, 744, and 882 or 884 (L2 and A2), 716, and 744 (L3 and A3), and 728, 744, and 760 (L4 and A4), identical spectra being obtained for plerocercoids and adult tapeworms (Fig. 2).

TABLE 1
Constituents of Cerebrosides from Plerocercoids of *Spirometra erinacei*[a]

Component	Subfractions			
	L1	L2	L3	L4
Hexose				
Galactose	69	99	100	100
Glucose	31	1	0	0
Sphingoid base				
d18:0[b]	100	41	57	21
t18:0[c]	0	59	43	79
Fatty acid				
16:0	0	10.6	21.8	0
18:0	4.6	20.8	31.2	31.5
18:1	0	2.8	0	0
26:0	17.2	9.3	0	0
26:1	4.5	3.4	0	0
27:0	3.7	1.9	0	0
27:1	2.7	2.5	0	0
28:0	13.5	9.3	0	0
28:1	36.5	30.3	0	0
30:1	12.8	6.8	0	0
18h:0[d]	0	0	40.2	61.4
Others	4.5	2.3	6.8	7.1

[a]Values are expressed as percentages of the total components.
[b]Sphinganine.
[c]4-D-Hydroxysphinganine (phytosphingosine).
[d]2-Hydroxy stearic acid.

TABLE 2
Constituents of Cerebrosides from Adult *Spirometra erinacei*[a]

Component	Subfractions			
	A1	A2	A3	A4
Hexose				
Galactose	88	93	93	87
Glucose	12	7	7	13
Sphingoid base				
d18:0[b]	100	56	57	33
t18:0[c]	0	44	43	67
Fatty acid				
16:0	5.5	17.7	11.0	0
18:0	11.4	37.2	18.5	15.3
18:1	4.0	1.0	0	0
26:0	27.3	11.6	0	0
26:1	3.3	1.4	0	0
27:0	2.7	1.1	0	0
27:1	3.3	0	0	0
28:0	6.0	16.8	0	0
28:1	24.2	8.2	0	0
30:1	8.5	3.6	0	0
18h:0[d]	0	0	65.8	83.6
Others	3.8	1.4	5.6	1.1

[a]Values are expressed as percentages of the total components.
[b]Sphinganine.
[c]4-D-Hydroxysphinganine (phytosphingosine).
[d]2-Hydroxy stearic acid.

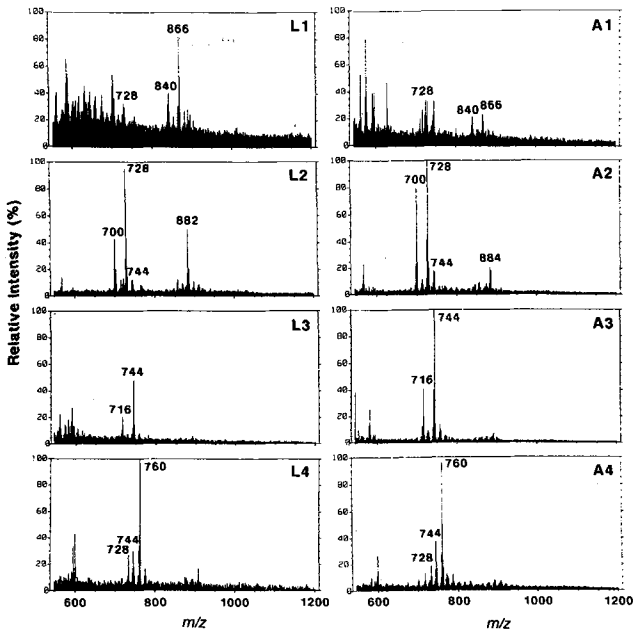


FIG. 2. Negative-ion fast atom bombardment mass spectrometry spectra of cerebroside subfractions from plerocercoids and adult tapeworms. L, plerocercoids; A, adult tapeworms.

Based on the HPLC/FAB–MS spectra (Fig. 2) and the analytical data for sphingoid bases and fatty acids (Tables 1 and 2), the major molecular species of ceramides were determined (Table 3). Selected peaks representing pseudo-molecular ions, such as *m/z* 700, 716, 728, 744, 760, 840, 866, 882, and 884, were also observed in the mass chromatograms obtained on micro HPLC/FAB–MS (data not shown), thus confirming the ceramide species shown in Table 3. In addition, the relative abundances of the respective molecular species determined by FAB–MS reflected those obtained on chemical analysis of ceramide moieties.

Considering the combination of ceramide species and the hexose composition, cerebroside molecules were slightly different between the two growth stages: glucocerebrosides of plerocercoids contained d18:0 and nonhydroxy fatty acids as ceramide constituents, whereas those of adult tapeworms contained various ceramide species, such as d18:0- or t18:0-linked to hydroxy or nonhydroxy fatty acids. These findings suggest that glucose can combine with not only the d18:0 but also the t18:0 sphingoid base in adult tapeworms.

TABLE 3
Major Molecular Species of Ceramides in Cerebroside Subfractions of *Spirometra erinacei*

	Subfractions			
m/z	L1 and A1	L2 and A2	L3 and A3	L4 and A4
700		d18:0–16:0		
716			t18:0–16:0	
728	d18:0–18:0	d18:0–18:0		d18:0–18:0
744		t18:0–18:0	d18:0–18h:0	t18:0–18:0
			t18:0–18:0	t18:0–18:0
760				t18:0–18h:0
840	d18:0–26:0			
866	d18:0–28:1			
882		t18:0–28:1 (L2)		
884		t18:0–28:0 (A2)		

The results of the present study demonstrate that the composition of *S. erinacei* cerebrosides is different from those of other cestode species. For example, *S. mansonoides* (6), a species taxonomically close to *S. erinacei*, contains three major cerebroside species, t18:0-18:0, d18:0-18h:0, and t18:0-18h:0. On the other hand, *M. coturnix* cerebroside contains d20:0 and t20:0 sphingoid bases, and longer chain hydroxy fatty acids (24h:0 and 26h:0) (11). The composition of *E. multilocularis* cerebroside fatty acids is different, since these acids include hydroxylated components ranging from C_{16} to C_{28} (8). In contrast to these cestode species, cerebrosides from *T. crassiceps* lack hydroxy fatty acids (7). We suggest that the distribution of cerebrosides in each of the various cestodes studies is an inherent property of that species, since the composition is not essentially different between *S. erinacei* plerocercoids and adults, although they are found in radically different hosts.

In summary, the cerebrosides from plerocercoids and the adult form of *S. erinacei* were separable into four subfractions. The different TLC mobilities were mainly due to differences in ceramide species. It should be noted that the two growth stages had essentially the same cerebroside constituents in spite of the entirely different environmental conditions; there were measurable differences in the quantitative distributions of the four cerebroside subfractions, i.e., in their hexose, fatty acid and sphingoid base substituents. These findings show that the glycolipids were homeostatically maintained as plasma membrane components throughout the lifecycle of this parasite, although slight modification of ceramide constituents may occur. In addition, the composition of sphingoid bases and fatty acids corresponded to that of SEGLx, a novel fucosylated glycosphingolipid that was recently reported by our group (5). This suggests that cerebrosides represent possible biosynthetic precursors of SEGLx.

ACKNOWLEDGMENTS

We wish to thank Dr. T. Udagawa for his encouragement throughout the course of this work. We also thank Dr. A. Suzuki (Tokyo Metropolitan Institute of Medical Science) for his interest in the study, and T. Taketomi (Shinshu University) for the gift of the standard fatty acids, 27:0 and 28:0. This work was supported in part by a Sasagawa Scientific Research Grant from the Japan Science Society, and by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Karlsson, K.A. (1989) *Ann. Rev. Biochem. 58*, 309–350.
2. Hakomori, S. (1990) *J. Biol. Chem. 265*, 18713–18716.
3. Hori, T., and Sugita, M. (1993) *Prog. Lipid Res. 32*, 25–45.
4. Dennis, R.D., and Wiegandt, H. (1993) *Adv. Lipid Res. 26*, 321–351.

5. Kawakami, Y., Nakamura, K., Kojima, H., Suzuki, M., Inagaki, F., Suzuki, A., Sonoki, S., Uchida, A., Murata, Y., and Tamai, Y. (1993) *J. Biochem. 114*, 677–683.
6. Singh, B.N., Costello, C.E., Levery, S.B., Walenga, R.W., Beach, D.H., Mueller, J.F., and Holz, Jr., G.G. (1987) *Mol. Biochem. Parasitol. 26*, 99–112.
7. Dennis, R.D., Baumeister, S., Geyer, R., Peter-Katalinic, J., Hartmann, R., Egge, H., Geyer, E., and Wiegandt, H. (1992) *Eur. J. Biochem. 207*, 1053–1062.
8. Persat, F., Bouhours, J.-F., Mojon, M., and Petavy, A.F. (1990) *Mol. Biochem. Parasitol. 41*, 1–6.
9. Persat, F., Bouhours, J.-F., Mojon, M., and Petavy, A.F. (1990) *Mol. Biochem. Parasitol. 38*, 97–104.
10. Persat, F., Bouhours, J.-F., Mojon, M., and Petavy, A.F. (1992) *J. Biol. Chem. 267*, 8764–8769.
11. Nishimura, K., Suzuki, A., and Kino, H. (1991) *Biochim. Biophys. Acta 1086*, 141–150.
12. Ando, S., Waki, H., and Kon, K. (1987) *J. Chromatogr. 405*, 125–134.
13. Kojima, H., and Tamai, Y. (1975) *Jpn. J. Exp. Med. 45*, 231–234.
14. Suzuki, M., Yamakawa, T., and Suzuki, A. (1991) *J. Biochem. 109*, 503–506.

[Received March 25, 1994, and in revised form January 13, 1995; Revision accepted January 19, 1995]

The Structure of the Core Polyol of the Ether Lipids from *Sulfolobus acidocaldarius*[1]

Akihiko Sugai[a,*], Rie Sakuma[a], Ikuko Fukuda[a], Norio Kurosawa[b], Yuko H. Itoh[b], Kazuo Kon[c], Susumu Ando[c] and Toshihiro Itoh[a]

[a]Division of Chemistry, College of Liberal Arts and Sciences, Kitasato University, Sagamihara, Kanagawa, 228 Japan, [b]Department of Bioengineering, Faculty of Engineering, Soka University, Hachioji, Tokyo 192, Japan and [c]Department of Membrane Biochemistry, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173, Japan.

ABSTRACT: The major ether-type lipid structures of *Sulfolobus acidocaldarius* (ATCC33909) were composed of caldarchaeol and calditoglycerocaldarchaeol. However, the characterization by nuclear magnetic resonance spectroscopy and mass spectrometry showed that the structure of calditol in calditoglycerocaldarchaeol is not nonitol, 2-(1′,2′,3′-trihydroxypropyl)1,2,3,4,5,6-hexahydroxyhexane, but 2-hydroxymethyl-1-(2,3-dihydroxypropoxy)2,3,4,5-cyclopentanetetraol with an ether linkage in the molecule. Such an intermolecular ether linkage was resistant to BCl_3 treatment, but nonresistant to 57% HI degradation treatment conducted at 100°C for 60 h, producing 2-hydroxymethyl-1,2,3,4,5-cyclopentanepentaol from calditol as reaction product. Further, it was confirmed that the structure of calditol is essentially a derivative of glycerol, and hydrocarbon chains were conjugated to the glycerol-like site in the structure. The calditol with an ether linkage in the molecule suggested an important role regarding the properties of heat-resistance and acid-resistance observed in *Sulfolobales*.
Lipids 30, 339–344 (1995).

The membranes of thermoacidophilic archaebacteria are monolayers based on two types of cyclic tetraether core lipids in which two polyols are linked together through two C_{40} isoprenoid chains (1–4). In one core type the hydrophilic portions were two glycerol units; the core has been termed caldarchaeol. The other type displayed hydrophilic portions of glycerol and calditol, and could be called calditoglycerocaldarchaeol according to Nishihara *et al.* (5). It was reported by De Rosa *et al.* (6) that the structure of calditol from calditoglycerocaldarchaeol of *Sulfolobus solfataricus* was nonitol, 2-(1′,2′,3′-trihydroxypropyl)-1,2,3,4,5,6-hexahydroxyhexane (hereafter, calditoglycerocaldarchaeol with the structure of nonitol will be described as nonitoglycerocaldarchaeol). Because no such nonitoglycerocaldarchaeol has been found in *Halobacteria* and *Methanobacteria* of *Archaea,* the presence of nonitoglycerocaldarchaeol in bacterial lipids has been considered as a specific taxonomic marker for *Sulfolobales* (7–10).

We have isolated several species of sulfur-dependent, extremely thermophilic archaebacteria from different hot springs in Japan and have investigated the structure of their bacterial lipids (A. Sugai, I. Fukuda and T. Itoh, unpublished data), comparing them with the type strains of *Sulfolobaceae.* From these experimental results, both caldarchaeol and calditoglycerocaldarchaeol were detected from *S. acidocaldarius* (ATCC33909), *Acidianus brierleyi* (IFO15269), *Metallosphaera sedula* (IFO15509), and our strains. In all these strains, calditoglycerocaldarchaeol was a major core component which constituted about 80% of all cores. However, the results of fast atom bombardment mass spectrometry (FAB–MS) analysis for calditoglycerocaldarchaeol prepared from these strains were not in agreement with those of the calculated molecular mass of nonitoglycerocaldarchaeol. From this finding it could be presumed that the structure of calditol obtained from our calditoglycerocaldarchaeol was different from the nonitol in nonitoglycerocaldarchaeol from *S. solfataricus.* On the other hand, in the early works of archaebacterial lipids, it was reported by Langworthy *et al.* (11) that the structure of calditol might be cyclic–polyol linked *via* an ether linkage to glycerol in *S. acidocaldarius.* In this paper we investigated the structure of calditol from calditoglycerocaldarchaeol in *S. acidocaldarius,* and we report that the structure of calditol obtained in our experiments is that of a new type polyol which was postulated by Langworthy *et al* (11).

[1]Presented at the international workshop on "Molecular Biology and Biotechnology of Extremophiles and Archaebacteria," Wako, Japan, August 1993.

*To whom correspondence should be addressed at Division of Chemistry, College of Liberal Arts and Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan.

Abbreviations: CI, chemicals ionization; DEPT, distortionless enhancement polarization transfer; DMSO, dimethyl sulfoxide; FAB–MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography/mass spectrometry; HMBC, ^{1}H-Detected multiple-bond connectivity; HPTLC, high-performance thin-layer chromatography; NBA, 3-nitrobenzylalcohol; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Materials. Chloroform and methanol were distilled before use. All other chemicals and solvents were of analytical grade.

Organisms and culture conditions. Sulfolobus acidocaldarius (ATCC33909) was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and the cells were grown at 75°C, pH 2.5 in 5-L medium consisting of inorganic salts [$CaCl_2 \cdot 2H_2O$, 0.4 g; $MgSO_4 \cdot 7H_2O$, 1.25 g; KH_2PO_4, 1.4 g; $(NH_4)_2SO_4$, 6.5 g], supplemented with 5 g of yeast extract and 10 g of sucrose. The cultures were aerobically grown for 60 h.

Lipid extraction. Total lipids were extracted from harvested cells with neutral solvents as follows: Cells were harvested from the culture medium by centrifugation, washed twice with 20 mL of distilled water, and suspended in 10 mL of methanol. After the addition of 5-mL chloroform, the mixture was sonicated for 20 min and centrifuged. The supernatant was then transferred to a recovery flask. The precipitated cells were suspended in 30 mL of chloroform/methanol/water (1:2:0.5, by vol) and sonicated for 20 min. Each suspension was centrifuged, and the supernatant was combined with that previously obtained. The extraction was repeated three times. All supernatants were dried using a rotary evaporator at 40°C. Residual lipids were dissolved by adding 5 mL of chloroform/methanol (2:1, vol/vol) and 0.5 mL of water with thorough mixing. Methanol was then added to the mixture until the solution was clear. The mixture was used for lipid analysis as a crude total lipid.

Thin-layer chromatography (TLC). TLC was performed with silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (0.25 mm; Merck, Darmstadt, Germany) which were activated at 120°C for 2 h before use. The TLC of polar lipids was conducted on activated plates that had been dipped in 3% H_3BO_3–methanol, and developed using chloroform/methanol/0.2% CaCl (55:45:7, by vol, solvent system A). The core lipids, including calditoglycerocaldarchaeol, were chromatographed on activated plates in chloroform/ethanol (9:1, vol/vol, solvent system B). Acetylated core lipids and nonitol were developed on activated plates in chloroform/ethyl acetate (9:1, vol/vol, solvent system C). Lipid spots on TLC plates were detected by spraying the Dittmer reagent (12) for phospholipids, and both reagents, for all lipids. After spraying with both reagents, all lipids were visualized as charred spots after heating at 160°C for 10 min. Preparative TLC for the purification of the lipids or their degradation products were also chromatographed on HPTLC plates with the same solvents.

Preparation of core lipids. Total bacterial lipids (10 mg) were heated under reflux in 1.5 mL of 5% methanolic–HCl in a stoppered reaction tube for 2 h to release the polar head groups from the intact lipids. The core lipids were obtained from the organic phase after partition between chloroform and water. The core lipids were chromatographed on a silica gel column (i.d. 15 mm × 300 mm) with Iatrobeads 6RS-8060 (Iatron, Tokyo, Japan). The column was eluted stepwise with solvents of increasing polarity from chloroform to chloroform/methanol at 9:1 (vol/vol), 4:1 (vol/vol), and 2:1 (vol/vol). Chloroform/methanol (4:1, vol/vol) eluted calditoglycerocaldarchaeol, which was further purified by preparative TLC with solvent B.

Preparation of calditol. The following procedure is a modification of the method of Nishihara and Koga (13). Calditoglycerocaldarchaeol (100 mg) was treated with 2 mL of BCl_3/chloroform (1:1, vol/vol) at room temperature for 3 h. The reaction solution was dried under a stream of nitrogen, and borate was removed by the repeated addition of methanol followed by drying. The residue was chromatographed on an Iatrobeads column (i.d. 5 mm × 50 mm) which was then eluted stepwise with solvents of increasing polarity from chloroform to chloroform/methanol at 2:1 (vol/vol), 3:2 (vol/vol) and 1:1 (vol/vol). The calditol fraction was eluted with chloroform/methanol (3:2, vol/vol).

Cleavage of the ether bond in calditol. Calditol (1 mg) was treated with 57% HI (0.5 mL) at 100°C for 72 h. After neutralizing the HI reaction mixtures with Amberlite CG-400I (OH^- form; Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan), the solution was filtered through glass wool and dried *in vacuo*. Following acetylation, the residual products were purified by column chromatography over Iatrobeads. Chloroform eluted the acetylated degradation products of calditol.

Periodate oxidation of calditoglycerocaldarchaeol. Calditoglycerocaldarchaeol (1 mg) was dissolved in 0.5 mL of chloroform, and 1.2 mL of methanol and 0.5 mL of 0.2M sodium metaperiodate were then added. The mixture was oxidized for 6 h at room temperature. After excess reagent was destroyed with glycerol, the oxidation products were recovered by extraction with chloroform (1 mL) and dried under a nitrogen stream. The residue was dissolved in 0.5 mL of chloroform, and 2 mL of methanol and 0.5 mL of 0.25M sodium borohydride were added. The reduction was performed for 15 h at room temperature. Excess borohydride was destroyed with 1M HCl. After drying, the reduction products were acetylated, and examined with gas chromatography/MS.

Methylation of calditol. The methylation of calditol was performed using the modified method of Ionel and Francisc (14) as follows: Calditol (1 mg) was dried under vacuum and methylated in a mixture of dimethyl sulfoxide (0.5 mL), CH_3I (0.1 mL), and NaOH (100 mg) powder under sonication for 10 min. The methylated calditol was then extracted with chloroform/water (1:1, vol/vol) under cooling, and purified by preparative TLC with solvent C.

Acetylation of calditol. A sample (10 mg) was dried under vacuum, and acetylated with 3 mL of acetic anhydride/pyridine (2:1, vol/vol) at 60°C for 12 h. After being cooled, the acetylated products were dried under a stream of nitrogen, and then purified by preparative TLC in the same manner as methylated calditol.

Trimethylsilation of calditol. The trimethylsilation of calditol was done using the mixture of pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane (5:1:1, by vol) at

100°C for 40 min. After cooling, the trimethylsilated calditol was extracted with chloroform/water (1:1, vol/vol).

MS analysis. Negative and positive FAB–MS of calditol was obtained using a JMS-AX505H (JEOL, Tokyo, Japan) instrument with a matrix of glycerol, and for acetylated calditol, 3-nitrobenzylalcohol (NBA), or a mixture of NBA and NaI was used as the matrix. GC/MS was performed on a glass packed column (3% OV-101, i.d. 3 mm × 1 m; GL Sciences, Tokyo, Japan) at a temperature increasing from 100 to 250°C at a rate of 8°C/min and 40 mL/min of isobutane chemical ionization reagent gas using a JMS-DX300 (JEOL) instrument.

Nuclear magnetic resonance spectroscopy (NMR) analysis. ^{1}H and ^{13}C NMR proton decoupled spectra were obtained using a Varian VXR-300 (Varian Instruments, Ltd., Palo Alto, CA) at 300 and 75 MHz, respectively. Calditol and acetylated calditol were dissolved in dimethyl sulfoxide (DMSO) or DMSO-d_6. Chemical shifts were given in ppm relative to tetramethylsilane as the internal standard. Analysis of the multiplicities of the peaks found in the ^{13}C NMR spectra of calditol and acetylated calditol were achieved using the distortionless enhancement by polarization transfer (DEPT) technique, which discriminates between methine, methyl, and methylene spins.

RESULTS

NMR data for calditol. The ^{1}H NMR spectrum of calditol showed the presence of seven hydroxyl groups. Their proton resonances could be seen at 4.18 ppm (triplet), 4.45 ppm (doublet), 4.46 ppm (triplet), 4.55 ppm (singlet and doublet), 4.65 ppm (doublet), and 5.01 ppm (doublet). These chemical shifts disappeared by adding DMSO-d_6. Also, the ^{13}C NMR spectrum of calditol obtained using the DEPT technique showed nine carbons at 61.83 ppm (methylene), 63.26 ppm (methylene), 70.43 ppm (methine), 72.54 ppm (methylene), 75.32 ppm (methine), 76.10 ppm (methine), 79.92 ppm (single), 81.54 ppm (methine), and 91.35 ppm (methine). The number of carbons was the same as in nonitol. Detailed information on the structure could not obtained from ^{1}H NMR of the intact calditol, as no assignments could be made, due to the ambiguous separations of the ^{1}H chemical shifts in a high magnetic field.

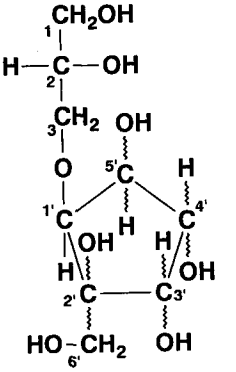
NMR data for acetylated calditol. In fully acetylated calditol, each of the seven ^{13}C chemical shifts of the methyl and carbonyl resonances derived from the acetyl groups was observed at 20.21–21.68 ppm and 168.81–170.17 ppm, respectively. No ^{1}H chemical shifts from hydroxy groups were observed in acetylated calditol. These results showed that all seven hydroxy groups observed in intact calditol were fully acetylated. In the region from 58.61 to 86.07 ppm, nine ^{13}C signals, due to the skeletal carbons of acetylated calditol, could be seen as the same number of skeletal carbons of intact calditol.

The ^{13}C NMR chemical shifts of individual carbons and ^{1}H NMR chemical shifts of protons in acetylated calditol are shown in Table 1. The postulated calditol structure is shown in Scheme 1. Assignments of ^{1}H to ^{13}C were made by the ^{1}H-detected multiple quantum coherence spectrum technique (Fig. 1A). The skeletal structure of acetylated calditol was confirmed by the ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence (HMBC) spectrum technique in which the relationship between a proton and 2–3 carbon bonds from the proton, $^2J_{CH}$ and $^3J_{CH}$, were observed. The results of HMBC are shown in Figure 1B: long-range coupling of ^{1}H and ^{13}C of acetylated calditol was observed from H-1 to C-2, H-2 to C-1, H-3 to C-1, C-2 and C-1′, H-1′ to C-3, C-2′, C-3′, and C-5′, H-3′ to C-1′ and C-5′, H-4′ to C-5′, H-5′ to C-1′, and C-4′, H-6′ to C-2′ and C-3′. The ^{1}H-^{1}H correlation spectroscopy showed cross peaks from H-1 to H-3 and H-1′ to H-3′, but no cross peak was observed between H-3 and H-1′ (data not shown). No linkages of the acetyl group to C-3 and C-1′ were observed by long-range selective proton decoupling and HMBC. Because neither C-3 nor C-1′ is hydroxylated, an ether linkage would appear to be present between these carbons, based on the C-3 and C-1′ in acetylated calditol being moved downfield (see Table 1).

TABLE 1
^{13}C Nuclear Magnetic Resonance Spectroscopy (NMR) Chemical Shifts and ^{1}H NMR Chemical Shifts for Acetylated Calditol[a]

Carbon number	^{13}C NMR δ_C	^{1}H NMR δ_H	
1	61.99	4.09	4.19
2	69.84	5.03	
3	69.60	3.73	3.74
1′	84.24	4.24	
2′	86.07	—[b]	
3′	71.82	5.36	
4′	71.98	5.22	
5′	78.65	5.30	
6′	58.61	4.34	4.71

[a]Carbon atoms were numbered as shown in Scheme 1.
[b]Absence of proton signal.



SCHEME 1

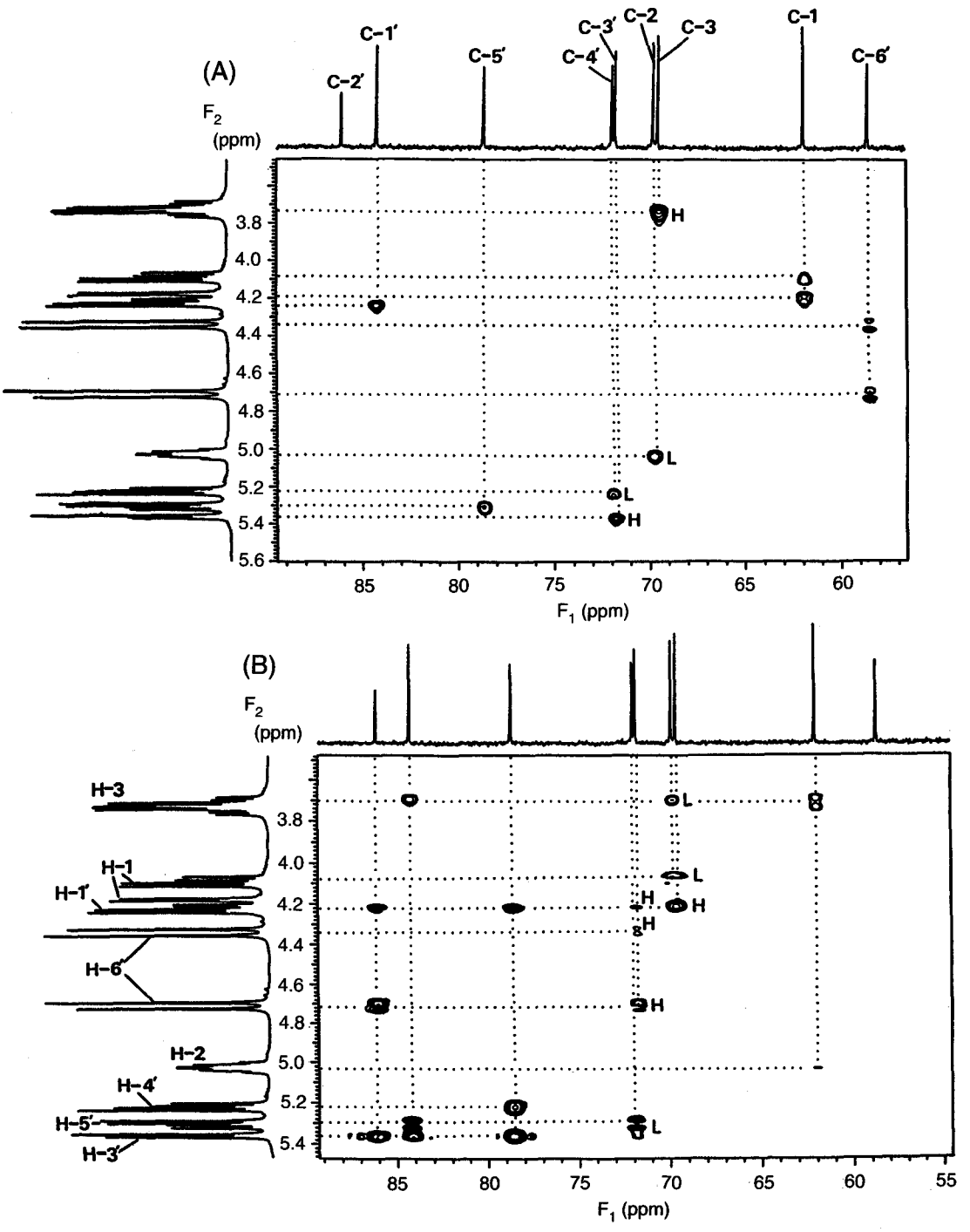


FIG. 1. (A) ^{1}H-Detected multiple-bond heteronuclear multiple quantum coherence spectrum and (B) ^{1}H-detected multiple quantum coherence spectrum of acetylated calditol. The axis of F_2 corresponds to ^{1}H nuclear magnetic resonance (NMR) chemical shifts and the axis of F_1 corresponds to ^{13}C NMR chemical shifts. "H" indicates a high magnetic field and "L" indicates a low magnetic field.

Nuclear Overhauser effects (NOE) were detected between H-1′ and H-5′ ($J_{H1'\text{-}H5'}$ = 5.25 Hz), H-3′ and H-4′ ($J_{H3'\text{-}H4'}$ = 5.25 Hz), respectively, indicating a *cis* relationship between these two protons. The existence of NOE between H-4′ and H-5′ ($J_{H4'\text{-}H5'}$ = 8.33 Hz) could not be examined, as those ^{1}H NMR chemical shifts were close to each other. H-1′ and C-6′ appeared to be in a *cis* conformation, because NOE was observed between H-1′ and H-6′. Based on NMR, the structure of calditol appeared to be **1**, 2-hydroxymethyl-1-(2,3-dihydroxypropoxy)-2,3,4,5-cyclopentanetetraol.

The mass spectra data of calditol. Negative FAB–MS of calditol showed a peak at *m/z* 253 [(M – H)$^-$; 100%], *m/z* 507 [(2M – H)$^-$; 56], *m/z* 761 [(3M – H)$^-$; 6] and *m/z* 1015 [(4M – H)$^-$; 2]. Positive FAB–MS showed a peak at *m/z* 255 [(M + H)$^+$; 100%], *m/z* 347 [(M + glycerol + H)$^+$; 16] and *m/z* 439 [(M + 2-glycerol + H)$^+$; 5]. In these FAB–MS spectra, a num-

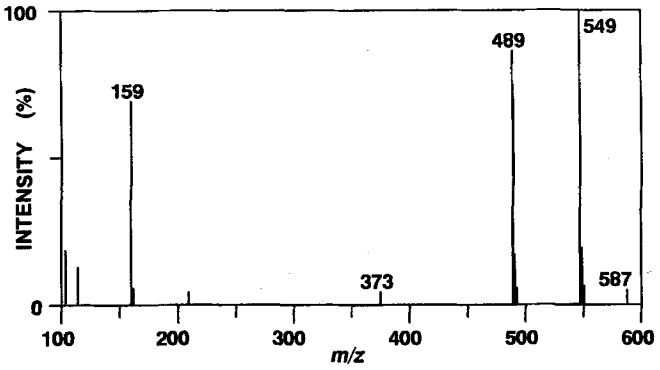


FIG. 2. Gas chromatography/mass spectrometry [chemical ionization (CI)] spectrum of acetylated calditol.

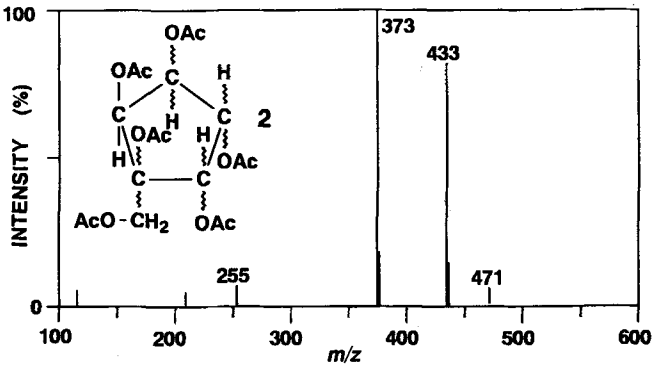


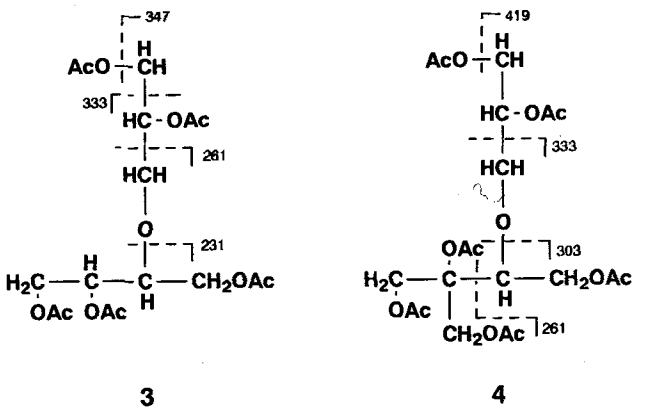
FIG. 3. Gas chromatography/mass spectrometry (CI) spectrum of the acetylated hydroiodic acid degradation product of calditol. A satisfactory conclusion could not be drawn from nuclear magnetic resonance.

ber of noncovalent cluster peaks were observed, but showed strong molecular ion peaks corresponding to the postulated molecular formula $C_9H_{18}O_8$; 254.

GC/MS of acetylated calditol showed a peak at *m/z* 549 as a molecular ion peak (Fig. 2), and methylated calditol and trimethylsilated calditol showed parent peaks at *m/z* 353 and *m/z* 759, respectively (data not shown). The molecular peaks of these calditol derivatives observed by GC/MS were confirmed by positive FAB–MS spectra. These results supported the estimated structure of calditol as determined by NMR, as these derivatives possessed seven acetyl, seven methyl, and seven trimethylsilyl groups, respectively.

The degradation of calditol. An attempt was made to cleave the intermolecular ether linkage to confirm the structure of calditol. After BCl_3 treatment of calditol was done under the general conditions (2,8), the products were acetylated and examined by GC/MS. From these results, only acetylated calditol was detected on GC/MS, so this linkage could not be cleaved using the BCl_3 method. The cleavage was accomplished by heating at 100°C for 60 h with 57% HI. The degradation products of calditol by 57% HI treatment were acetylated and analyzed by GC/MS. Acetylated 1,2-dihydroxy-3-iodopropane was not detected as a degradation product, but a peak corresponding to acetylated 1-hydroxymethyl-1,2,3,4,5-cyclopentanpentaol was obtained, and the mass spectrum gave peaks for $(M + K)^+$ at *m/z* 471, the protonated molecule, $(M + H)^+$ at *m/z* 433, and a peak at *m/z* 373 due to the loss of one acetyl group from structure **2** in Figure 3.

The structure of calditoglycerocaldarchaeol. Periodate oxidation of calditoglycerocaldarchaeol was carried out to confirm the conjugated sites of the hydrocarbon chains to calditol in calditoglycerocaldarchaeol, and, after reducing, the polyol moiety was recovered by BCl_3 treatment. The polyols were acetylated and examined with GC/MS. The result showed that the glycerol portion of calditoglycerocaldarchaeol was not decomposed and was detected quantitatively, though no calditol was detected because of complete cleavage. Two major peaks were observed on the gas chromatogram of the calditol degradation products. The proportions of the two peaks, A and B, were 0.6:0.4. The GC/MS of A and B were as follows: A, *m/z*

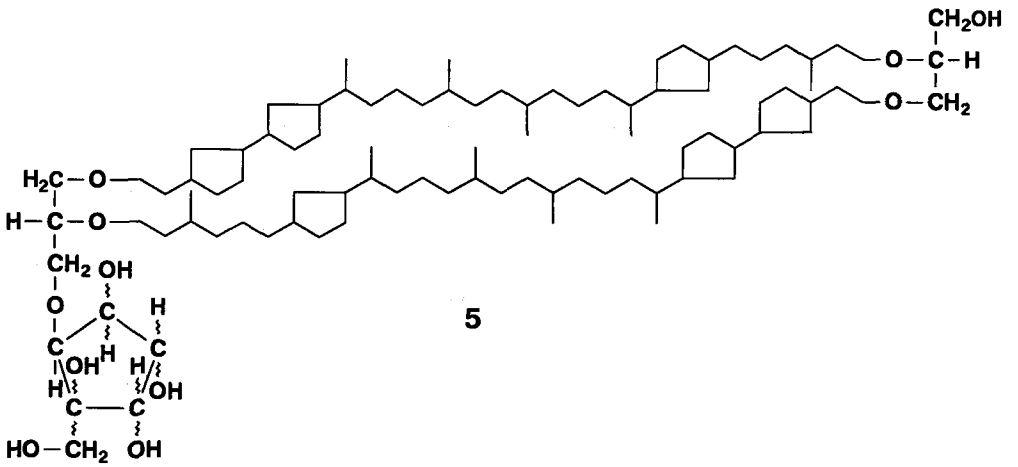


SCHEME 2

406 (M + H; 7%), 347(100), 333(1), 303(2), 261(6), 231(11), 159(61); and (B), *m/z* 478 (M + H; 9%), 419(100), 333(5), 303(7), 261(3), and 159(73). This result suggested that structures A and B were **3** and **4**, respectively, as shown in Scheme 2. Because both structures showed that the glycerol-like portion of calditol had not been oxidized, it seems most likely that the C_{40} isoprenoid chains had been conjugated to C-1 and C-2. From these results, the structure of calditoglycerocaldarchaeol was deduced to be that indicated in Scheme 3. Also, the molecular ion peaks (producing plural molecular ions by the number of cyclopentane rings in alkyl chains), *m/z* 1456 and *m/z* 1452, on negative FAB–MS of calditoglycerocaldarchaeol prepared from *S. acidocaldarius*, suggested the structure of **5**.

DISCUSSION

In the present work, we have described the structural elucidation of calditol from the core lipid of *S. acidocaldarius*. The existence of an ether linkage in the calditol molecule was determined by NMR data. The intermolecular ether linkage of calditol was resistant to BCl_3 treatment, but nonresistant to HI degradation treatment. The HI degradation procedure produced 1-hydroxymethyl-1,2,3,4,5-cyclopentanepentaol from



SCHEME 3

calditol. This resistance was presumably due to the existence of a steric hindrance by hydroxy groups in the polyol. The structure of calditol was determined as heptanol, **1**, 2-hydroxymethyl-1-(2,3-dihydroxypropoxy)-2,3,4,5-cyclopentanetetraol. However, respective NOE observed in between H-1′ and H-2′, H-1′ and H-6′, were very weak, so we could not completely exclude the possibility that no NOE were presented in them. If the existence of those NOE are in error, all orientations of H-2′, H-3′, H-4′, and C-6′ on the cyclopentane ring of calditol should be contrary to **1**. To confirm this structure, it would be necessary to examine the chemical structure of calditol by X-ray diffraction studies.

The stereochemical structure of the glycerol moiety of calditol was not unambiguously established by the values of $J_{H1\text{-}H2}$ (6.45 Hz) and $J_{H2\text{-}H3}$ (4.50 Hz), and the NOE data. But, if the 2,3-dihydroxypropyl group of calditol is assumed to be present instead of glycerol, hydrocarbon chains should bond to the *sn*-2,3 configuration of the glycerol moiety through ether linkages, as can he seen in other archaebacterial lipid cores (8). In any case, the structure obtained in our experiments shows that calditol from *S. acidocaldarius* is not just a peculiar polyol, as was the previously published nonitol from *S. solfataricus,* but is essentially a derivative of glycerol. Also, our structure of calditoglycerocaldarchaeol, **5**, as shown in Scheme 3, is a stable form of glycolipid since a hexose-like compound is conjugated with glycerol through an ether linkage.

In a historical note, the structure of calditol was shown by Langworthy *et al.* (11) to be the cyclic–polyol linked *via* an ether linkage to glycerol, and the note indicated six free hydroxy groups in the structure of calditoglycerocaldarchaeol. Our presented structures **1** and **5** were neither nonitol nor nonitoglycerocaldarchaeol, respectively, but satisfied the results reported by Langworthy *et al.* (11). This structure may be one of the factors attributable to the properties of heat-resistance and acid-resistance observed in *Sulfolobales*.

ACKNOWLEDGMENTS

This work was supported in part by the Sasagawa Scientific Research Grant (5-136) from the Japan Science Society, and a grant from the Kitasato Research Fund (H5-12).

REFERENCES

1. De Rosa, M., Gambacorta, A., Nicolaus, B., Sodano, S., and Bu'Lock, J.D. (1980) *Phytochemistry 19,* 833–836.
2. De Rosa, M., Gambacorta, A., Nicolaus, B., Chappe, B., and Albrecht, P. (1983) *Biochim. Biophys. Acta 753,* 249–256.
3. De Rosa, M., Gambacorta, A., Nicolaus, B., and Bu'Lock, J.D. (1980) *Phytochemistry 19,* 821–825.
4. De Rosa, M., Esposito, E., Gambacorta, A., Nicolaus, B., and Bu'Lock, J.D. (1980) *Phytochemistry 19,* 827–831.
5. Nishihara, M., Morii, H., and Koga, Y. (1987) *J. Biochem. 101,* 1007–1015.
6. De Rosa, M., De Rosa, S., Gambacorta, A., and Bu'Lock, J.D. (1980) *Phytochemistry 19,* 249–254.
7. Trincone, A., Lanzotti, V., Nicolaus, B., Zillig, W., De Rosa, M., and Gambacorta, A. (1989) *J. Gen. Microbiol. 135,* 2751–2757.
8. Langworthy, T.A. (1986) in *Thermophiles: General, Molecular, and Applied Microbiology* (Brock, T.D., ed.) pp. 107–135, John Wiley & Sons, New York.
9. Langworthy, T.A. (1985) in *The Bacteria, Vol. VIII: Archaebacteria* (Woese, C.R., and Wolfe, R.S., eds.) pp. 459–497, Academic Press, New York.
10. Lo, S., Montague, C.E., and Chang, E.L. (1989) *J. Lipid Res. 30,* 944–949.
11. Langworthy, T.A., Mayberry, W.R., and Smith, P.F. (1974) *J. Bacteriol. 119,* 106–116.
12. Dittmer, J.C., and Lester, R.L. (1964) *J. Lipid Res. 5,* 126–127.
13. Nishihara, M., and Koga, Y. (1988) *J. Lipid Res. 29,* 384–388.
14. Ionel, C., and Francisc, K. (1984) *Carbohydr. Res. 131,* 209–217.
15. De Rosa, M., De Rosa, S., and Gambacorta, A. (1977) *J-S. Chem. Comm.* 514–515.

[Received April 24, 1993, and in revised form February 18, 1995; Revision accepted February 22, 1995]

Role of Cyclic Adenosine Monophosphate in Phospholipid Synthesis in *Mycobacterium smegmatis* ATCC 607

Harpinder Kaur and G.K. Khuller*

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160012, India

ABSTRACT: The present study was undertaken to examine the influence of intracellular levels of cyclic AMP on phospholipid synthesis in *Mycobacterium smegmatis* ATCC 607. The cyclic AMP levels were modulated by growing cells in the presence of activator (forskolin) and inhibitor (atropine) of adenylate cyclase, the synthesizing enzyme of cyclic AMP. Forskolin-grown cells exhibited a 1.4-fold increase in the level of cAMP while a similar decrease (1.8-fold) was seen with atropine-grown cells. These altered levels of cAMP in turn affected the total content, composition and synthesis of phospholipids. Total phospholipid content increased and decreased in cells grown in the presence of forskolin and atropine, respectively. These observations were further supported by alterations in [^{14}C]acetate incorporation as well as in activities of glycerol kinase and glycerol-3-phosphate acyltransferase, the key enzymes of phospholipid synthesis. Protein phosphorylation mechanism seems to be involved in phospholipid metabolism as the activities of protein kinase increased and decreased in cells grown in forskolin or atropine cells. Our results demonstrate a correlation between phospholipid synthesis and intracellular levels of cAMP in *M. smegmatis*.
Lipids 30, 345–349 (1995).

Cyclic AMP participates in cellular function both in prokaryotic and eukaryotic organisms (1,2). Cyclic AMP in different mycobacterial species is present in large amounts in cells and culture fluid as reported earlier (3). Much of the evidence of cAMP involvement in regulation of various processes is based on the methods involving manipulation of its intracellular levels (4). In *Mycobacterium smegmatis*, this nucleotide has been shown to enhance the incorporation of [^{14}C]acetate into lipids when the cells were incubated with dibutryl-cAMP (5) or forskolin (6) while in *M. phlei*, no relationship was observed between the changes in phospholipid composition and cAMP (7). However, no systematic study has been carried out to demonstrate whether a correlation exists between intracellular levels of cAMP and lipid biosynthesis in mycobacteria. Therefore, the present study has been carried out with forskolin and atropine, activator/inhibitor of adenylate cyclase to find out the relationship between cAMP and lipid biosynthesis in *M. smegmatis* ATCC 607. Further, preliminary experiments were also carried out to determine the role of cyclic AMP-dependent protein phosphorylation in relation to phospholipid metabolism.

MATERIALS AND METHODS

[1-^{14}C]Glycerol-3-phosphate (specific activity 4.75 mCi/mmol^{-1}, [^{3}H]ATP (specific activity 13.5 Ci/mmol), [^{3}H]adenosine 3′,5′-cyclic monophosphate (specific activity 2000 mCi/mmol), [γ-^{32}P]ATP (specific activity 3000 Ci/mmol), and [1-^{14}C]acetate (specific activity 56.2 mCi/mmol) were purchased from Bhabha Atomic Research Centre (Bombay, India). [^{3}H]Glycerol (specific activity 2.5 Ci/mmol) was obtained from Amersham (Buckinghamshire, United Kingdom). Atropine, forskolin, cyclic AMP-dependent protein kinase inhibitor (catalogue number P-5015) and acetyl CoA were obtained from Sigma (St. Louis, MO). Other chemicals used were of analytical grade.

Organism and growth conditions. Mycobacterium smegmatis ATCC 607 (M. 607) obtained from NTCC (London, United Kingdom) was grown in modified Youman's medium as a static culture at 37°C (8). Cells were harvested in their mid log-phase and processed for different studies.

Determination of optimum concentration of atropine and forskolin. Mid log-phase cells were incubated with different concentrations of atropine (2 to 10 mM) and forskolin (1–7 μM) in Krebs Ringer buffer (KRB) (pH 7.2) for 3 h at 37°C. The cells were collected by centrifugation at 4°C, and a homogenate for adenylate cyclase assay was prepared by sonicating the cells at 4°C in 40 mM Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 5,000 × *g* for 20 min to remove cell debris, and the supernatant was used for enzyme assay. The activity of adenylate cyclase was assayed by the method of Salomon *et al.* (9).

Extraction and estimation of intracellular cAMP. Cells (0.3–0.5 g wet weight) were suspended in hot 0.1N HCl, and the suspension was kept in a boiling water bath for 10 min. Each sample was chilled, centrifuged at 12,000 × *g* for 10 min

*To whom correspondence should be addressed.

Abbreviations: CL, cardiolipin; KRB, Kreb's Ringer buffer; PE, phosphatidylethanolamine; PIM, phosphatidylinositol mannosides.

Copyright © 1995 by AOCS Press

at 4°C, and pH of the supernatant was adjusted to 7.0. The supernatant was again centrifuged at 12,000 × *g* for 10 min to remove precipitated material (10). Intracellular cAMP was then measured by the method of Takeda *et al.* (11).

Quantitation of lipids. Lipids were extracted by the method of Folch *et al.* (12). Phospholipids were quantitated by the method of Marinetti (13). Total phospholipids were separated from total lipids by thin-layer chromatography in acetone while individual phospholipids were separated by one-dimensional thin-layer chromatography on silica gel H (14) impregnated with 0.18% ammonium sulfate in chloroform/methanol/7N ammonia (65:25:4, by vol) as developing solvent. The respective fractions were scraped off and transferred into vials containing a toluene-based scintillation fluid for radioactivity determination.

Uptake studies. Cells grown in the presence of optimum concentration of atropine (6 mM) or forskolin (5 μM) were harvested and suspended in KRB (pH 7.2). [^{14}C]Acetate (25 μCi/100 mL) pulse was given for different time intervals ranging from 15 to 90 min. After appropriate incubation, cells were filtered and lipids were extracted.

Fatty acid synthesis. Fatty acid synthesis by intact cells was studied according to the method of Buttke and Ingram (15). [^{14}C]Acetate was added to the control, forskolin, and atropine grown cultures, at a concentration of 10 μCi/g cells/15 mL KRB buffer and incubated at 37°C for 60 min. The cells were filtered, washed with normal saline and lipids were extracted by the Folch procedure (12). Transesterification of lipids was done using 2% H_2SO_4 in methanol by the method of Silbert *et al.* (16). The methyl esters were extracted with pentane and concentrated under vacuum prior to analysis. Radioactivity was counted using a toluene-based scintillation fluid.

Enzyme assays. The homogenate for adenylate cyclase, phosphodiesterase and glycerol-3-phosphate acyltransferase was prepared by sonicating cells at 4°C in 40 mM Tris-HCl buffer (pH 7.5). For glycerol kinase, the cells were homogenized in 10 mM Tris-HCl buffer (pH 8.0) with 250 mM sucrose. The homogenate for protein kinase activity assay was prepared in 50 mM-Tris maleate buffer (pH 7.0). The homogenates were centrifuged at 5,000 × *g* for 20 min to remove cell debris, and the resulting supernatants were used for enzyme assays.

The activities of adenylate cyclase and phosphodiesterase were measured according to the methods of Salomon *et al.* (9) and Aboud and Burger (17), respectively. For the activity of acyltransferase, the assay system used was of Okuyama *et al.* (18), and the filter paper disc method of Goldfine (19), as modified by van den Bosch and Vagelos (20), was employed. The activity of glycerol kinase was determined by the method of Kasinathan *et al.* (21). The activity of protein kinase was measured as described by Banerjee and Sarkar (22).

RESULTS

The role of cAMP in phospholipid synthesis of *M. smegmatis* was examined by modulating its intracellular cAMP levels with the help of forskolin and atropine. These compounds alter the activity of adenylate cyclase and thus increase or decrease the cAMP levels in the cell.

Initially, the optimum concentrations of both forskolin and atropine were determined by measuring the activity of adenylate cyclase in the presence of different concentrations of either atropine (0–10 mM) or forskolin (0–7 μM) in the assay mixture. The activity of adenylate cyclase increased with increasing concentration of forskolin while the reverse was observed in case of atropine (Table 1). Maximum increase (56%) and decrease (48%) in adenylate cyclase activity was observed at 5 μM of forskolin or 6 mM of atropine, respectively. Therefore, these concentrations were used for further studies.

After determining the optimum concentration, cells were grown in the presence of these concentrations, and the growth was monitored. No significant change in the growth pattern of *M. smegmatis* was observed with optimum concentration of forskolin (5 μM) and atropine (6 mM). To examine the effect of cAMP on phospholipid synthesis, levels of intracellular cAMP were determined in cells grown in the presence of these compounds. Increased levels of cyclic AMP (40%) was exhibited in forskolin grown cells while a decrease (44%) was observed in atropine-grown cells (Table 2). These altered levels of cyclic AMP were probably due to the effect of these compounds on adenylate cyclase as the activity of this enzyme increased (60%) and decreased (45%) respectively in cells grown with forskolin or atropine (Table 2). However, the activity of phosphodiesterase remained largely unaltered by either treatment.

The effect of altered levels of cAMP on phospholipids of *M. smegmatis* was examined (Table 3). It was found that total content of phospholipids increased in forskolin-grown cells while a decrease was observed in atropine-grown cells. On examining the individual phospholipid components, significant increases in phosphatidylinositol mannosides (PIMs) and cardiolipin (CL) were observed in cells grown with forskolin while all the phospholipid components (PIMs, phos-

TABLE 1
Effect of Forskolin and Atropine on Adenylate Cyclase Activity of *Mycobacterium smegmatis* ATCC 607[a]

	Adenylate cyclase (nmol/mg protein/min)		
mM Atropine		μM Forskolin	
2	16.21 ± 0.55[c]	1	23.00 ± 0.41[b]
4	14.07 ± 0.67[d]	2	25.27 ± 0.40[d]
6	10.87 ± 0.12[d]	3	27.61 ± 0.92[d]
8	11.64 ± 0.39[d]	4	31.65 ± 0.68[d]
10	13.27 ± 0.52[c]	5	33.14 ± 1.19[d]
		6	30.03 ± 0.77[d]
		7	27.98 ± 0.82[d]
Control	20.73 ± 1.20		

[a]Values are mean ± SD of three (atropine) or four (forskolin) different batches.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

TABLE 2
Activities of cAMP Metabolizing Enzymes and Intracellular Levels of cAMP of *Mycobacterium smegmatis* ATCC 607 Grown in the Presence of Atropine or Forskolin[a]

Cells	Adenylate cyclase (nmol/mg protein/min)	Phosphodiesterase (nmol/mg protein/min)	Intracellular cAMP levels (nmol/g dry wt)
Control	20.87 ± 2.32	1.54 ± 0.10	18.67 ± 1.31
Atropine	11.17 ± 1.25[c]	1.79 ± 0.05[b]	10.54 ± 1.21[c]
Forskolin	32.74 ± 3.06[c]	1.30 ± 0.22[b]	25.94 ± 1.98[c]

[a]Values are mean ± SD of three different batches grown for 4 d in the presence of 6 mM atropine or 5 μM forskolin.
[b]Not significant.
[c]$P < 0.01$.

TABLE 3
Phospholipid Composition of *Mycobacterium smegmatis* ATCC 607 Grown in the Presence of Atropine or Forskolin[a]

Cells	Total phospholipids (mg/g dry wt)	PIM	PE (mg/g dry wt)	CL
Control	25.42 ± 1.13	9.21 ± 0.78	4.64 ± 0.42	11.33 ± 0.68
Atropine	19.25 ± 1.01[c]	6.63 ± 0.68[c]	3.89 ± 0.24[b]	9.21 ± 0.68[c]
Forskolin	30.79 ± 0.99[c]	11.48 ± 0.79[b]	5.20 ± 0.54[d]	14.16 ± 1.02[b]

[a]Values are mean ± SD of five different batches grown for 4 d in the presence of atropine (6 mM; 5 batches) or forskolin (5 μM; 3 batches).
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]Not significant; PIM, phosphatidylinositol mannosides; PE, phosphatidylethanolamine; CL, cardiolipin.

phatidylethanolamine and CL) decreased in atropine-grown cells. These changes in the phospholipids were further confirmed by examining the rate of incorporation of [^{14}C]acetate into phospholipids and total lipids (Fig. 1). A net stimulation in the rate was observed in forskolin-grown cells as compared to control cells while the rate was decreased in the atropine-grown cells.

The levels of phospholipids in cells change only when the activity of either synthesizing or degradative enzymes are altered. The activities of glycerol kinase and glycerol-3-phosphate acyltransferase, the key enzymes of phospholipid synthesis, increased and decreased in forskolin and atropine-grown cells, respectively (Table 4). Further, the activity of glycerol-3-phosphate acyltransferase depends upon the synthesis of fatty acids. Therefore, *in vivo* fatty acid synthesis was monitored, and it was found to increase (34%) in forskolin-grown cells and decrease (46%) in atropine-grown cells (Table 5).

In order to study the mechanism of action of cAMP, the phosphorylating capacity of *M. smegmatis* was examined. A crude mycobacterial cell extract phosphorylated histone, indicating the presence of protein kinase activity (Table 6). On addition of different amounts of cAMP to the assay mixture, the activity of protein kinase increased with increasing concentration of cyclic AMP. There was maximum activation at 2 μM, indicating the presence of cAMP-dependent protein kinase in *M. smegmatis* (Fig. 2). The protein kinase activity was completely inhibited in the presence of specific cAMP-dependent protein kinase inhibitor (20–60 μg/μL), further indicating its presence in *M. smegmatis*. The phosphorylating activity of forskolin- and atropine-grown cells was increased and decreased, respectively, compared to controls (Table 7), which may be due to the increased and decreased intracellular levels of cAMP in these cells (Table 2).

DISCUSSION

In prokaryotes, the role of cyclic AMP in the synthesis of inducible enzymes is well established but its effect on the regulation of phospholipid metabolism is not known. In this study, the influence of cyclic AMP on phospholipid synthesis was determined by modulating the intracellular levels of cAMP in *M. smegmatis*. Cells grown with forskolin and atropine exhibited increased and decreased levels of cAMP, respectively (Table 2). The raised levels of cAMP in forskolin-grown cells was due to the activation of adenylate cyclase by forskolin (Table 2) which is known to bind the catalytic subunit of the enzyme (23). Atropine decreased the activity of adenylate cyclase (Table 2), but its mechanism is unknown. Forskolin has been shown to increase the cAMP levels in human platelets (24) while atropine decreased its levels in *Neurospora crassa* (4).

Forskolin, which increased the cAMP levels, further enhanced the total content and synthesis of phospholipids while the opposite was seen with atropine, which lowered the levels of cAMP (Table 3). These results show a direct correlation between intracellular cAMP and phospholipid synthesis in *M. smegmatis* similar to the observations in *M. gypseum*

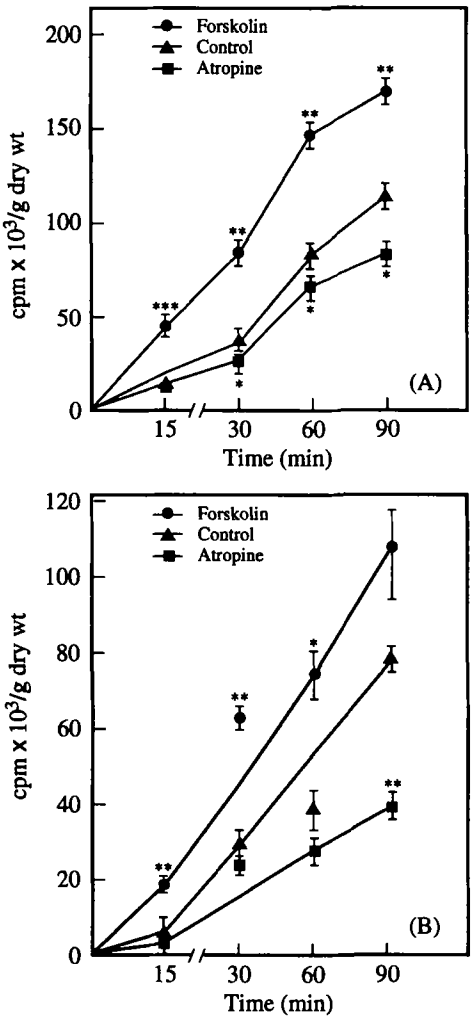


FIG. 1. Incorporation of [^{14}C]acetate into total lipids (A) and phospholipids (B) extracted from control cells (▲), forskolin (●), atropine (■) grown cells. After suspending the cells in Krebs Ringer buffer, [^{14}C]acetate (25 μCi/100 mL buffer) was added, and aliquots were taken at different time intervals ranging from 0–90 min. Lipids were extracted, and the radioactivity incorporated into lipids was counted as described in the Materials and Methods section. The values are mean ± SD of three different batches. Asterisk denotes values that are significantly different from control. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ based on Student's *t*-test.

TABLE 4
Activities of Enzymes of Phospholipid Biosynthesis of *Mycobacterium smegmatis* ATCC 607 Grown in the Presence of Atropine or Forskolin[a]

Enzyme	Control	Atropine	Forskolin
Glycerol kinase (nmol/mg protein/h)	319.30 ± 12.99	129.28 ± 4.40[c]	521.50 ± 17.66[c]
Glycerol-3-phosphate (nmol/mg protein/h)	26.01 ± 3.06	11.58 ± 3.00[b]	56.41 ± 5.27[c]

[a]Values are mean ± SD of three different batches grown for 4 d in the presence of 6mM atropine or 5 μm forskolin.
[b]$P < 0.01$.
[c]$P < 0.001$.

TABLE 5
Fatty Acid Synthesis by *Mycobacterium smegmatis* ATCC 607 Grown in the Presence of Atropine or Forskolin[a]

Cells	Incorporation of [^{14}C]acetate into total fatty acids (cpm/g dry wt)
Control	8654 ± 983
Forskolin	11630 ± 1041[b] (+34%)
Atropine	4630 ± 484[c] (–46%)

[a]Values are mean ± SD of three different batches grown for 4 d in the presence of 6 mM atropine or 5 μM forskolin. Values in parentheses indicate percent increase or decrease.
[b]$P < 0.05$.
[c]$P < 0.01$.

TABLE 6
Protein Kinase Activity in *Mycobacterium smegmatis* ATCC 607[a]

Cells	Protein kinase (pmol/mg protein/min)
Control	87.3 ± 7.31
Control + inhibitor[b]	No activity
Forskolin	139.2 ± 12.01[c]
Atropine	52.3 ± 3.5[c]

[a]Values are mean ± SD of four different batches.
[b]cAMP-dependent protein kinase inhibitor (conc. 20–60 μg/200 μL).
[c]$P < 0.001$.

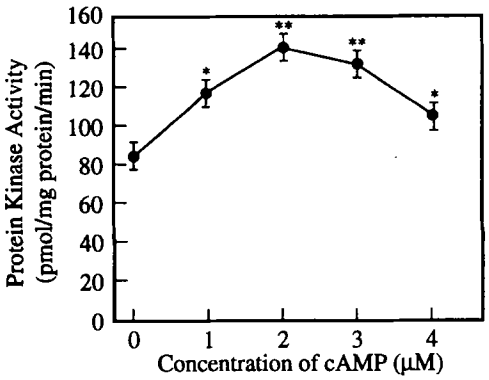


FIG. 2. Effect of cAMP on protein kinase activity in cell extracts of *Mycobacterium smegmatis*. Protein kinase activity was measured by comparing the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to histone in the presence of different concentration of cAMP. Assay was carried out in 50 mM Tris-maleate buffer (pH 7.0), containing 20 mM $MgCl_2$, 0.25 mg histone, 150 μM [γ-^{32}P]ATP, 10 mM DTT, 2.5 mg/mL bovine serum albumin, and an appropriate amount of enzyme preparation. Values are mean ± SD of four different batches. Asterisk denotes values that are significantly different from control (without cAMP). *$P < 0.05$, **$P < 0.01$ based on Student's *t*-test.

(25). The earlier report (5) that dibutryl cAMP increased the incorporation of [^{14}C]acetate into lipids of *M. smegmatis* further supports our observations with forskolin.

The increased and decreased activities of glycerol kinase and glycerol-3-phosphate acyltransferase in forskolin- and atropine-grown cells (Table 4) also correlates with the altered synthesis of phospholipids in these cells. We observed that

the activity of these enzymes depended on cAMP, which is consistent with earlier reports (24,26). Further, the activity of glycerol-3-phosphate acyltransferase depends upon the synthesis of fatty acids, which also was found to exhibit increases or decreases in cells grown in forskolin or atropine (Table 5). These results are in agreement with the earlier observation (3) that dibutryl cAMP increased the incorporation of [^{14}C]acetate into fatty acids by *M. smegmatis*. Thus, the increased or decreased synthesis of fatty acids in response to the altered levels of cAMP may ultimately be responsible for the altered synthesis of phospholipids.

In eukaryotes, action of cAMP is mainly mediated through cAMP-dependent protein kinase (27). As *M. smegmatis* is believed to lack cAMP receptor protein (28), the possibility of the presence of protein kinase in this organism was examined. A crude extract of the mycobacteria was found to possess protein kinase activity by its phosphorylation of histone (Table 6). This has also been demonstrated in other bacterial species (29). The addition of exogenous cAMP enhanced the protein kinase activity, indicating that at least some of the protein kinase present in *M. smegmatis* is cAMP-dependent. Similar findings were reported earlier for *Escherichia coli* (30), *Streptococci* (31), and *Saccharomyces cerevisiae* (32). Increased or decreased activity of protein kinase in cells grown in forskolin or atropine, respectively (Table 6), is probably due to the altered expression of the protein in mycobacteria, and this aspect warrants further study.

In summary, we can conclude from the present results that phospholipid biosynthesis in *M. smegmatis* is influenced by cyclic AMP and that its effects are probably mediated through protein kinases.

REFERENCES

1. Pall, M.L. (1981) *Microbiol. Rev. 45*, 462–480.
2. Botsford, J.L., and Harman, J.G. (1992) *Microbiol. Rev. 56*, 100–122.
3. Padh, H., and Venkitasurbramanian, T.A. (1977) *Life Sciences 20*, 1273–1280.
4. Scott, W.A., and Solomon, B. (1975) *J. Bacteriol. 122*, 454–463.
5. Ahmad, S., Murthy, M.V.V.S., and Venkitasurbramanian, T.A. (1981) *FEMS Microbiol. Letters 12*, 261–263.
6. Sareen, M., Kaur, H., and Khuller, G.K. (1993) *J. Biosci. 18*, 207–212.
7. Reddy, P.H., Burra, S.S., and Murthy, P.S. (1992) *Can. J. Microbiol. 38*, 339–342.
8. Youmans, A.S., and Karlson, A.G. (1947) *Am. Rev. Tuberc. 55*, 529–535.
9. Salomon, Y., Loros, C., and Rodbell, M. (1974) *Anal. Biochem. 58*, 541–548.
10. Hylemon, P.B., and Phibbs, P.V. (1974) *Biochem. Biophys. Res. Commun. 60*, 88–95.
11. Takeda, T., Kuo, T., Shuntoh, H., and Tanaka, C. (1989) *J. Biochem. 105*, 327–329.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–507.
13. Marinetti, G.V. (1962) *J. Lipid Res. 3*, 1–11.
14. Kates, M. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T.S., and Work, E., eds.) Vol. 3, pp. 502–579, North Holland Publishing Company, Amsterdam.
15. Buttke, T.M., and Ingram, L.O. (1978) *Biochemistry 17*, 5282–5286.
16. Silbert, D.F., Ulbright, T.M., and Honeggar, J.L. (1973) *Biochemistry 12*, 164–171.
17. Aboud, M., and Burger, M. (1971) *Biochem. Biophys. Res. Commun. 43*, 173–182.
18. Okuyama, H., Kankura, T., and Nojima, S. (1977) *J. Biol. Chem. 252*, 6682–6686.
19. Goldfine, H. (1966) *J. Lipid Res. 7*, 146–149.
20. van den Bosch, H., and Vagelos, P.R. (1970) *Biochim. Biophys. Acta 218*, 233–248.
21. Kasinathan, C., Chopra, A., and Khuller, G.K. (1982) *Lipids 17*, 859–869.
22. Banerjee, C., and Sarkar, D. (1990) *J. Gen. Microbiol. 136*, 1051–1057.
23. Daly, J.W. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17*, 81–89.
24. Imai, A., Hattori, H., Takahashi, M., Nakashima, S., Okano, Y., Hattori, T., and Nozawa, Y. (1984) *Thromb. Res. 35*, 539–549.
25. Bindra, A., and Khuller, G.K. (1992) *Biochim. Biophys. Acta 1124*, 185–189.
26. Argilaga, C.S., Russel, R.L., and Heimberg, M. (1978) *Arch. Biochem Biophys. 190*, 367–372.
27. Gancedo, J.M., Major, M.J., and Eraso, P. (1985) *TIBS 10*, 210–212.
28. Padh, H., and Venkitasurbramanian, T.A. (1980) *Microbios 27*, 69–78.
29. Chiang, T.M., Reizer, J., and Beachey, E.H. (1989) *J. Biol. Chem. 264*, 2957–2962.
30. Kuo, J.F., and Greengard, P. (1969) *J. Biol. Chem. 244*, 3417–3419.
31. Khandelwal, R.L., Spearman, T.N., and Hamilton, I.R. (1973) *FEBS Lett. 31*, 246–250.
32. Kinney, A.J., Myongsuk, B.L., Ponghol, S.S., Kelley, M.J., Gaynor, P.M., and Carman, G.M. (1990) *J. Bacteriol 172*, 1133–1136.

[Received April 4, 1994, and in final revised form January 18, 1995; Revision accepted January 19, 1995]

Purification and Properties of an Extracellular Lipase from the Fungus *Botrytis cinerea*

Pascal Comménil[a], Lionel Belingheri[b,*], Michel Sancholle[b] and Bertrand Dehorter[b]

[a]Laboratoire de Biologie et Physiologie Végétale, Université de Reims, 51062 Reims Cedex, France and [b]Laboratoire de Cryptogamie-Phytopathologie, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

ABSTRACT: An extracellular lipase (EC 3.1.1.3) from the fungus *Botrytis cinerea* has been purified to homogeneity and characterized. The purification included ammonium sulfate fractionation and sequential column chromatography. The purification of the preparation was 31-fold and recovery yield was 21%. The purified enzyme was associated with esterase activity according to activity staining on polyacrylamide gel. The molecular weight was determined as 60 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and estimated at 72 kDa using gel filtration, which suggests that the enzyme may be a monomer. The isoelectric point was 6.5, and optimal activity was obtained at 38°C and pH 6.0. This lipase showed a high specificity for synthetic substrates containing long-chain unsaturated fatty acids using umbelliferone esters. The effect of β-cyclodextrin on the hydrolysis of olive oil has been studied. The specific activity was 25 μmole/min/mg in the absence of β-cyclodextrin and 132 μmole/min/mg in its presence.
Lipids 30, 351–356 (1995).

Lipases (EC 3.1.1.3, glycerol ester hydrolase) are recognized as being important industrial enzymes. Their potential for industrial application was reviewed in 1989 (1). These enzymes are able to hydrolyze acylglycerols in aqueous medium and catalyze the synthesis of esters in organic medium (2,3). In the first group, lipases hydrolyze fats and oils by breaking the fatty acyl ester bonds of triacylglycerols (TGs). Lipases can be specific to the nature of the fatty acid on the glycerol backbone. Some are known to show preference for saturated fatty acids (4), while others preferred unsaturated fatty acids with a *cis*-double bond at the 9-position (palmitoleic, oleic, linoleic and linolenic acids) (5,6).

Lipases are produced by animals, plants, and microorganisms. To date, the majority of lipases used for biotechnological purposes have been isolated from fungi (7,8). Filamentous fungi are preferred sources of lipases, as they produce extracellular enzymes. The most productive species belong to the genera *Geotrichum*, *Aspergillus*, *Rhizopus*, *Mucor*, and *Penicillium* (9).

Botrytis cinerea is a ubiquitous pathogen, causing a serious disease in wine grapes *(Vitis vinifera).* It has the ability to produce a lipase. This enzyme, which is released into the culture medium, hydrolyzes the esters of unsaturated long-chain fatty acids. We used a sensitive fluorimetric method for measuring lipase activity using various esters of umbelliferone (UMB) (10).

In this paper, the purification and properties of an inducible extracellular lipase from *B. cinerea* are reported.

MATERIALS AND METHODS

Materials. UMB, fatty acid chlorides, Concanavalin A-Sepharose, methyl-α-D-mannopyranoside, bovine serum albumin, molecular weight markers for SDS–polyacrylamide gel electrophoresis (PAGE) (SDS6-H and SDS7) and for gel permeation were obtained from Sigma Chemical Co. (St. Louis, MO). Octyl-Sepharose CL-4B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Bio-Beads SM-2, silver stain kit, isoelectric focusing standards, and reagents for PAGE were obtained from Bio-Rad Laboratories (Richmond, CA). Tetrahydrofuran, pyridine, diethyl ether, ethylene glycol monomethyl ether (EMMEG), and other solvents were of chromatographic grade.

Lipase production. Botrytis cinerea was isolated from grapevines. The fungus was grown in 25/200-mm tubes containing 15 mL culture medium. The medium contained, per liter of distilled water, was: aspartic acid, 1.99 g; dextrose, 1 g; KH_2PO_4, 0.8 g; $CaHPO_4$, H_2O, 0.2 g; $MgSO_4$, $7H_2O$, 0.5 g; $MnSO_4$, $5H_2O$, 0.01 g; $CuSO_4$, $5H_2O$, 0.005 g; $FeSO_4$, $7H_2O$, 0.0001 g; methyl oleate, 3 g; and taurocholic acid, 0.6 g. The medium was adjusted to pH 5.5. Inoculum was prepared from seven day-old mycelial mats, broken by shaking with glass beads in 60 mL sterile water. Inocula of 2 mL were transferred to 1-L conical flasks containing 200 mL culture medium and incubated on an orbital shaker at 120 rpm. Cultures were harvested after seven days' growth at 23°C in darkness, at which time extracellular lipase activity was maximal.

Lipase purification. Culture fluid containing extracellular enzymes was separated from mycelia by filtration. Proteins

*To whom correspondence should be addressed at Laboratoire de Cryptogamie, Université des Sciences et Technologies de Lille, Bâtiment SN 2, 59655 Villeneuve d'Ascq Cedex, France.

Abbreviations: DG, diacylglycerol; EMMEG, ethylene glycol monoethyl ether; FFA, free fatty acids; MG, monoacylglycerol; pI, isoelectric point; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TG, triacylglycerol; TLC, thin-layer chromatography; UMB, umbelliferone.

Copyright © 1995 by AOCS Press

were precipitated from the culture filtrate with ammonium sulfate (51% saturation). The solution was constantly stirred at 4°C during the addition of ammonium sulfate. After standing 10 h at 4°C, the precipitate was collected by centrifugation at 30,000 × *g* for 20 min, dissolved in 10 mM phosphate buffer pH 7.0 (buffer A) and dialyzed against the same buffer overnight. After dialysis, ammonium sulfate was added to make the final 1 M concentration. The dialyzate was placed on an Octyl-Sepharose CL-4B column (11.5 × 1 cm) which had been equilibrated with buffer B (buffer A + 1 M ammonium sulfate). The column was washed with buffer B, then with buffer A, and finally with buffer C (buffer A + 0.3% polyoxyethylene 10-tridecyl ether, Emulphogene BC-720; Sigma). This is a nonionic detergent very similar to Triton X-100, but nonabsorbing at 280 nm (11). The lipase activity was found in the fraction eluted with buffer C. The passage on Bio-Beads SM-2 (column, 4 × 1 cm) allowed a partial removal of the detergent. The active fraction was applied to Concanavalin A-Sepharose column (4 × 1 cm) equilibrated with buffer A. The column was washed sequentially with buffer A, buffer A containing 0.1 M, then with 0.5 M methyl-α-D-mannopyranoside. Lipase was eluted with buffer A containing 0.1 M methyl-α-D-mannopyranoside, and the eluate was dialyzed against buffer A. The active fraction was concentrated by ultrafiltration (ultrafree-MC PTGC, 10 kDa cut-off; Millipore, Bedford, MA). The enzyme fraction was submitted to a Sephacryl S-200 column (98 × 1.5 cm) previously equilibrated with buffer A. All steps were performed at room temperature (20–25°C), and all the protein elution profiles were recorded spectrophotometrically at 280 nm.

Lipase assay. Lipase activity was assayed by a fluorimetric method (10) using umbelliferyl oleate as a standard substrate. The reaction mixture contained 20 µL of umbelliferyl oleate solution (4 mM) in EMMEG in a total volume of 1 mL of hydrochloride Tris–maleate buffer (0.05 M, pH 6.0). At time zero, enzyme fraction was added to this mixture, and the fluorescence emission (470 nm) was recorded over 2 min (F-1200 fluorescence spectrophotometer; Hitachi Instruments, Tokyo, Japan) at an excitation wavelength (320 nm) corresponding to the absorption peak of UMB. The reaction was carried out at 38°C. This method can be carried out quickly, in two to three minutes.

Lipolytic activity was also quantitated by a titrimetric assay using emulsion olive oil as substrate. Composition of the reaction mixture was 3 mL 0.1 M citrate/Na_2HPO_4, buffer pH 6.0, 16.5 mM $CaCl_2$ and 0.11 mM sodium taurocholate, 5 mL deionized water, 1 mL olive oil emulsion (50% vol/vol olive oil), and 1 mL of the enzyme preparation appropriately diluted with 0.1 M citrate/Na_2HPO_4 buffer. Incubations were carried out at 38°C. After 60 min, the reaction was stopped by the addition of 10 mL an ethanol/acetone mixture (1:1, vol/vol). The liberated fatty acids were titrated with 50 mM KOH. A unit of lipase specific activity was defined as the release of one micromole of fatty acid per min and per mg under the assay conditions.

The course of triolein (0.1 mL triolein in 5 mL reaction mixture as described above) hydrolysis by the lipase was followed by thin-layer chromatography (TLC). Diethyl ether was added to stop the reaction and to extract the products. Samples were taken to run TLC on plates (0.2 mm) of silica 60 F_{254} (E. Merck, Darmstadt, Germany). Development was with a hexane/diethyl ether/glacial acetic acid mixture (78:20:4, by vol). Spots were revealed with primulin and identified by comparison of their migration rates with those of known standards (Sigma).

Effects of metal ions, pH, and temperature on lipase activity. To determine the effects of metal ions on lipase activity, the enzyme was incubated in Tris–maleate buffer containing the agents to be tested. Reagents were added to an enzyme solution, and the enzyme activity was measured after 30 min pre-incubation at 38°C. The following buffers (50 mM) were used for the determination of the pH dependence on the enzyme activity: sodium citrate–phosphate (pH 4, 5, 6, 7); Tris–maleate (pH 6, 7, 8); Tris–HCl (pH 7, 8, 9); glycine–NaOH (pH 9, 10). The effect of pH on enzyme activity was analyzed after pre-incubation in buffered solutions for 30 min at 38°C. Corrections were made to compare lipolytic activities at different pH values as the fluorescent intensity of UMB varies with pH. The optimum temperature for enzyme activity was determined by incubation of the reaction mixture at different temperatures. The enzyme solution was pre-incubated for 30 min to various temperatures (5–60°C).

Substrate specificity of lipase activity. The specificity of the lipase for laurate, myristate, palmitate, stearate, oleate, and linoleate was calculated from the initial rate of hydrolysis of the respective umbelliferyl esters (80 µM). Fatty acid esters were synthesized according to the method in Reference 10.

Molecular weight determination. Native molecular weight was estimated by chromatography on a Sephacryl S-200 column (98 × 1.5 cm). The following proteins were used as standard markers: phosphorylase a (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The void volume was determined with blue Dextran 2000. Molecular weight of the purified lipase was also determined by SDS–PAGE according to the method of Laemmli (12) and resolved in 7.5–15% linear gradient polyacrylamide gel. The run was carried out at 24 mA. Proteins were detected in gels by silver staining (13). The molecular weight of the lipase was determined by reference to the mobilities of known molecular mass from SDS standards (Sigma).

Isoelectric point (pI) determination. Isoelectric focusing was performed on 5% polyacrylamide gels. A vertical minigel system with 8 × 7 cm cell format was utilized (Mini Protean; Bio-Rad) according to the method of Robertson *et al.* (14). Electrophoresis was carried out at 4°C for 1.5 h at 200 V constant voltage, then increased to 400 V constant voltage for an additional 1.5 h. Proteins were detected with silver stain. The pI of the lipase was estimated by comparison of its mobility to those of standard proteins (IEF; Bio-Rad) included on gel.

Activity staining. Native PAGE was performed on an 8% gel, and the esterase activity was identified by staining with

50 mg Fast blue RR salt, 30 mg α-naphthyl acetate in 2 mL acetone/water (1:1, vol/vol) and 80 mL Tris–maleate (0.1 M) at pH 6.4 (15).

Protein determination. Protein concentrations were determined according to Bradford (16) using the Bio-Rad protein assay kit. Bovine serum albumin was used as the standard in the assay.

RESULTS AND DISCUSSION

Enzyme purification. Botrytis cinerea produced an inducible extracellular lipase in a medium containing methyl oleate as inducer. Many lipases are considered to be inducible enzymes, their synthesis being promoted by their substrates, products of their hydrolysis, or substrate analogs. Dextrose (1 g/L) must be present in the culture medium in order to start the growth; a higher concentration results in a lower lipase production. When methyl oleate was not added, the activity was not detectable. Shaking improves the production of the lipase, probably because of the better oxygenation of the culture medium, and also results in a higher release of the lipase. Due to the great dilution of enzymes in the medium, a preliminary protein concentration was necessary. The lipase was precipitated from the culture supernatant at 51% ammonium sulfate saturation with 30% of total proteins. Chromatography of lipase on Octyl Sepharose is shown in Figure 1. There were strong hydrophobic interactions between the enzyme and the gel matrix which could be broken only by addition of detergent. Total lipase activity is eluted with 0.3% Emulphogene BC-720. Other detergents were assayed but, due to tailing and poor recovery of activity, Triton X-100 and sodium deoxycholate were discarded. Fractions 46–52 were pooled and, after passage on Bio-Beads SM-2, applied to the Concanavalin A-Sepharose column. The lipase activity was associated with the peak of proteins eluted by 0.1 M methyl-α-D-mannopyranoside (Fig. 2). This result suggests the glycosylation of the lipase. A glycosylation level of 5–10% appears to be fairly typical of fungal lipases (17). Fractions 21–24 were pooled, dialyzed, concentrated, and applied to a Sephacryl S-200 column. The complete purification achieved is shown in Table 1. A sixfold purification with 51% recovery of activity was obtained with the Octyl-Sepharose column. The purification protocol (Table 1) resulted in a 31-fold purification with a 21% recovery of total activity. This preparation is stable for at least several months at –20°C.

The purified lipase was homogeneous as shown by SDS–PAGE (Fig. 3) and IEF (Fig. 4). A single band was also detectable by esterase activity stain on polyacrylamide gel.

Enzyme characterization. The molecular weight of the lipase, calculated by SDS–PAGE, was 60 kDa (Fig. 3). The native molecular weight of the lipase was estimated by gel filtration through Sephacryl S-200 and was found to be 72 kDa. The lipase, therefore, consists of a single polypeptide chain. Most fungal extracellular lipases are monomers having molecular weights ranging from 30 to 64 kDa. The molecular mass was 30.3 kDa for *R. delemar* (18), 56 kDa for *P. simplicissimum* (19), and 64 kDa for *G. candidum* (5). The lipase from *Neurospora crassa* is a dimer with 27 kDa molecular weight subunits (20) and those from *Pythium ultimum* (6), a tetramer with 68 kDa subunits.

The molecular mass of the lipase, determined by gel filtration, was 12 kDa larger than that found by SDS–PAGE. This larger molecular weight may be an overestimate of the true mass, as gel filtration has been shown to overestimate the mass of another lipase (21). An initial attempt by using the gel filtration at the first step of the purification process was abandoned because of a tendency of the lipase to aggregate as a broad fraction on the column. Aggregation of lipases occurs often with mammalian (22) and microbial lipases (23). Lipases can also associate with glycosylated impurities present in the extract, leading to the formation of high-molecular weight aggregates.

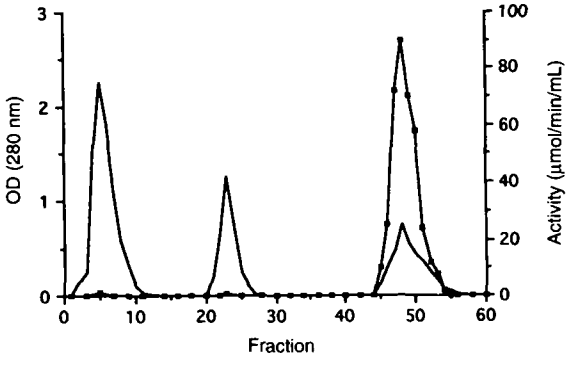


FIG. 1. Chromatography on Octyl-Sepharose (Pharmacia, Uppsala, Sweden). The enzyme solution after ammonium sulfate precipitation was loaded onto an Octyl-Sepharose column (11.5 × 1 cm). Protein (–) was measured by absorbance (OD) at 280 nm, and 3-mL fractions were collected. Lipase activity (–■–) starting at fraction 32, that eluted with detergent (0.3% Emulphogene BC-720; Sigma, St. Louis, MO) was measured spectrofluorimetrically as described in the Materials and Methods section.

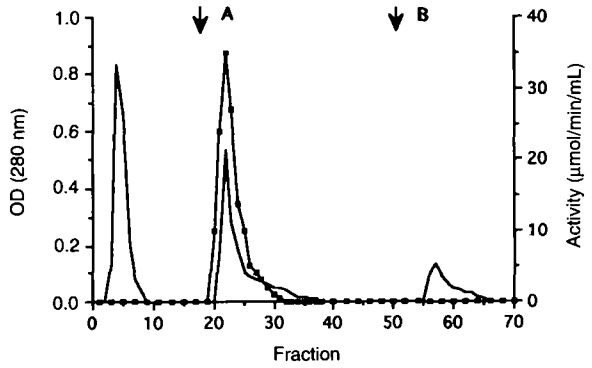


FIG. 2. Chromatography on Concanavalin A-Sepharose (Sigma, St. Louis, MO). Active fractions from the Octyl-Sepharose (Pharmacia, Uppsala, Sweden) column were applied on the column (4 × 1 cm). Elution conditions are given in the Materials and Methods section; 1-mL fractions were collected. Bound proteins were eluted with buffer containing 0.1 M methyl-α-D-mannopyranoside (A) and 0.5 M methyl-α-D-mannopyranoside (B). Shown are lipase activity (–■–) and protein (–).

TABLE 1
Purification of *Botrytis cinerea* Extracellular Lipase

Steps	Protein (mg)	Activity[a] (μmol UMB/min)	Specific activity[a] (μmol UMB/min/mg)	Yield (%)	Purification factor
Culture filtrate	15.5	1271	82	100	1
$(NH_4)_2SO_4$ precipitation	4.7	799	170	62.9	2.1
Octyl Sepharose CL-4B[b]	1.3	647.4	498	51	6.1
Con A Sepharose[c]	0.36	475.2	1320	37.4	18
Sephacryl S200[b]	0.106	272.8	2574	21.5	31.4

[a]Lipase activity was assayed by a fluorimetric method using umbelliferyl oleate as a standard substrate. Activity is defined as micromoles of umbelliferone (UMB) released per min at 38°C and per mg protein for specific activity. Details regarding the activity determinations are given in the Materials and Methods section.
[b]From Pharmacia Fine Chemicals, Uppsala, Sweden.
[c]Concanavalin-A Sepharose, from Sigma Chemical Co., St. Louis, MO.

The pI, determined by isoelectric focusing of the lipase in polyacrylamide gel was found to be about 6.5 (Fig. 4). pI of lipases from *R. delemar* (18), *G. candidum* (17), and *P. simplicissimum* (19) were 8.6, 4.0–4.7, and 4.1, respectively.

Effects of temperature, pH, and metal ions on lipase activity. Lipase activity is temperature-sensitive. Maximum activity occurred at 38°C, with 75% of the value exhibited between 32–40°C. The enzyme was completely inactivated above 60°C. The lipase proved to be quite stable at room temperature. After 48 h at room temperature, 98% of the initial activity remains in the control.

The pH dependence of the activity of the *B. cinerea* lipase was observed over the pH range 4.5–9.0, but the optimum pH was 6.0. The enzyme exhibited 70% or more of maximal activity between pH 5.5 and 6.5. The fact that the lipase is maximally active near its pI value may indicate that the binding of this enzyme to its hydrophobic substrate is best when its net charge is zero. Most of extracellular fungal lipases have acidic or neutral pH optima, and very few are alkaline lipases.

Actions of some cations were studied on lipase activity. Neither Ca^{2+} (5, 10, and 30 mM), Mg^{2+} (10, 50, and 100 mM), Na^+ (0.1, 0.5, and 1 M), nor K^+ (0.1, 0.5, and 1 M) had an effect on the action of the enzyme. Calcium ions are

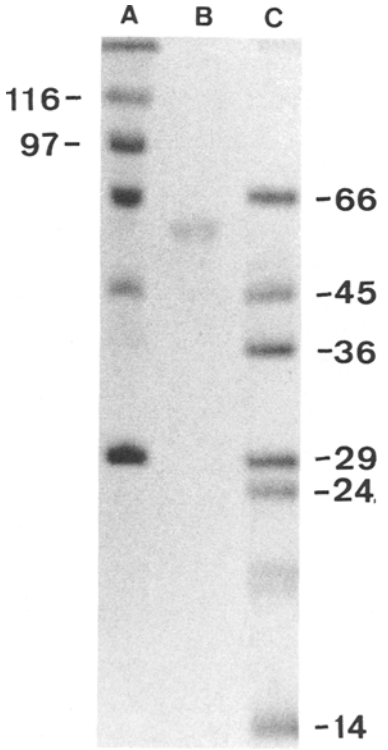


FIG. 3. SDS–polyacrylamide gel electrophoresis of purified lipase from *Botrytis cinerea* with silver staining. Lanes A and C, molecular weight standards; lane B, purified lipase. The masses of the molecular weight marker proteins (kDa) are indicated in margins: β-galactosidase (116 kDa); phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); glyceraldehyde-3-P-dehydrogenase (36 kDa); bovine carbonic anhydrase (29 kDa); trypsinogen (24 kDa); and α-lactalbumin (14 kDa).

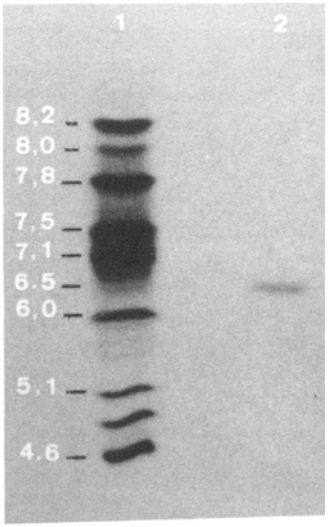


FIG. 4. Isoelectric focusing of the purified lipase from *Botrytis cinerea* with Coomassie blue staining. Lane 1, Standard protein preparation; lane 2, purified lipase. The isoelectric point values of the standard proteins are indicated in the left margin: lentil lectin (8.2), (8.0), (7.8); human hemoglobine C (7.5); human hemoglobine A (7.1); human carbonic anhydrase (6.5); bovine carbonic anhydrase (6.0); β-lactoglobulin B (5.1); and phycocyanin (4.6).

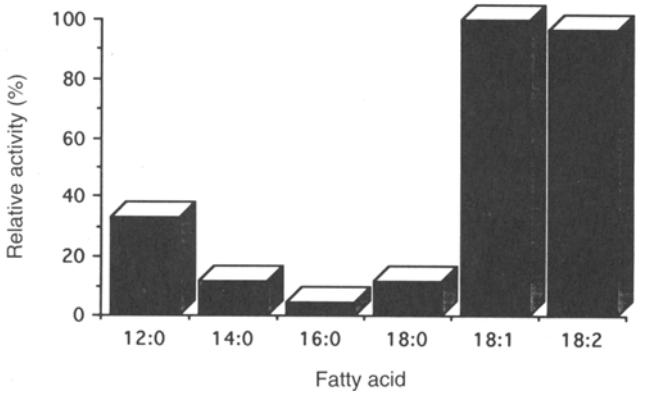


FIG. 5. Substrate specificity of lipase activity. Activities are expressed relative to that of the most active substrate.

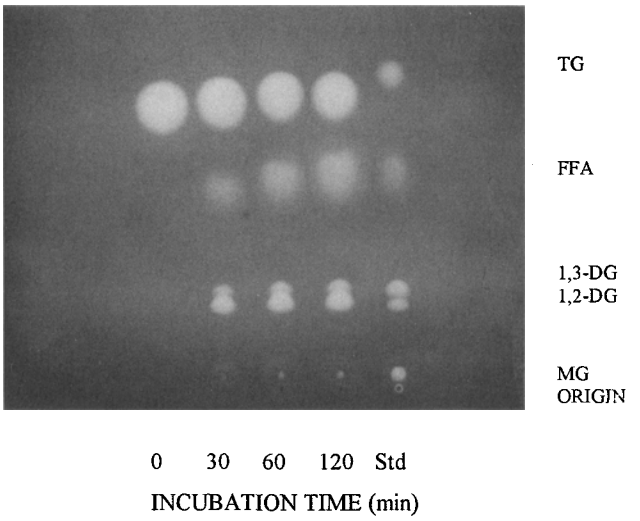


FIG. 6. Thin-layer chromatography of products of triolein hydrolysis by extracellular lipase from *Botrytis cinerea*. The reaction conditions are given in the Materials and Methods section. TG, triacylglycerol; 1,3-DG, 1,3-diacylglycerol; 1,2-DG, 1,2-diacylglycerol; FFA, free fatty acid; MG, monoacylglycerol.

known to increase lipase activity (24) by acting directly on the enzyme (25). They also can reduce the solubility of fatty acids generated during hydrolysis (26), which can be a major problem when using titrimetric methods. This problem was avoided by the fluorimetric method.

Substrate specificity of lipase activity. A comparison of the lipase activities toward different fatty acid ester substrates demonstrated important differences. The best hydrolysis rates were obtained with the oleic acid and linoleic acid esters (Fig. 5). The rate of hydrolysis for lauric acid ester is 33% of oleic acid ester, while it is only 12% for stearic acid and 5% for palmitic acid esters. In the C18 series, the presence of double bonds seems to enhance apparent lipase activity. We can then say that this lipase is very specific for the esters of unsaturated fatty acids. In the series of saturated fatty acids tested, maximum activity was found for C12. Gargouri *et al.* (27) demonstrated that enzyme activity toward triglycerides may reflect the dependence of the enzyme on appropriate surface tension and viscosity, which vary with fatty acid chain length and the number of double bonds, but does not show real substrate specificity.

TABLE 2
Effect of β-cyclodextrin on the Activity of the Extracellular Lipase from *Botrytis cinerea*

β-Cyclodextrin content[a]	Activity[b] (U/mg protein)	Relative activities[c]
Control (none)	25.03 ± 4.49[d]	100
0.5% (wt/vol)	71.85 ± 5.96[e]	287
1% (wt/vol)	93.24 ± 12.88[f]	372
1.5% (wt/vol)	132.60 ± 9.66[g]	530

[a]β-Cyclodextrin was added to the reaction mixture before activity determination.
[b]Each value is the mean ± SD of four determinations. One unit is defined as one micromole of fatty acid release per min from emulsified olive oil at 38°C and pH 6.0. Details regarding the activity determinations are given in the Materials and Methods section. Significance was assessed by Student's *t*-test: $P < 0.01$ between d and e, and $P < 0.05$ between e and f and f and g.
[c]Values were calculated relative to the control (without additive) using mean.

Use of a fluorimetric method is convenient for the rapid detection of lipases. In order to confirm that we measured a true lipase activity rather than a general esterase one, we checked the hydrolysis of olive oil. The specific activity of the purified enzyme was 25 µmole/min/mg. The lipase from *B. cinerea* is able to hydrolyze soluble esters (characteristic of esterases) as well as long-chain TG (natural substrate of lipases).

The effect of β-cyclodextrin (cycloamylose) on the activity of the lipase was studied using olive oil as substrate. A marked stimulation of the activity was observed by the addition of β-cyclodextrin to the assay mixture (Table 2). Optimal lipase activity (132 µmole/min/mg) was found with a concentration of 1.5% (wt/vol). The β-cyclodextrin was insoluble up to this concentration. The use of cyclodextrin in enzymology is known to enhance the solubility of hydrophobic compounds in water (28).

Positional specificity. Figure 6 shows a thin-layer chromatogram of the enzyme reaction products obtained after various reaction times (30, 60, and 120 min) with triolein as the substrate. The chromatoplate showed that 1,2 (2,3)-diolein was formed in larger quantities than 1,3-diolein. These results indicate that the enzyme hydrolyzes 1- and 3-positioned ester bonds in preference to 2-positioned ester bonds. This specificity is typical of the fungal lipases studied previously (6). The production rate of monoglycerides was lower than that of diacylglycerides.

These results are the first report of the purification and the characterization of an extracellular lipase from the fungus *B. cinerea*. Further work will generate information about the amino acid composition, *N*-terminal amino acid sequence, and the neutral sugar content of the lipase.

ACKNOWLEDGMENT

This work was supported by the Europol'Agro of Champagne-Ardenne through a grant to Pascal Commenil.

REFERENCES

1. Harwood, J., and Macrae, A.R. (1989) *Trends Biochem. Sci. 14*, 125–126.
2. Pororske, L.H. (1984) *J. Am. Oil Chem. Soc. 61*, 1758–1760.
3. Macrae, A.R., and Hammond, R.C. (1985) *Biotechnol. Gen. Eng. Rev. 3*, 193–217.
4. Hoshino, T., Sasaki, T., Watanabe, Y., Nagasawa, T., and Yamane, T. (1992) *Biosci. Biotech. Biochem. 56*, 660–664.
5. Baillargeon, M.W., and McCarthy, S.G. (1991) *Lipids 26*, 831–836.
6. Mozaffar, Z., and Weete, J.D. (1993) *Lipids 28*, 377–382.
7. Iwai, M., and Tsujisaka, Y. (1984) in *Lipases* (Borgström, B., and Brockman, H.L., eds.), pp. 443–466, Elsevier, Amsterdam.
8. Macrae, A.R. (1983) in *Microbial Enzymes and Biotechnology* (Fogarty, W.M., ed.) pp. 225–250, Applied Science Publishers, New York.
9. Godtfredsen, S.E. (1990) in *Microbial Enzymes and Biotechnology,* 2nd edn. (Fogarty, W.M., and Kelly, C.T., eds.) Vol. 7, pp. 225–274, Elsevier, New York.
10. de Laborde de Montpezat, T., de Jeso, B., Butour, J.L., Chavant, L., and Sancholle, M. (1990) *Lipids 25*, 661–664.
11. Furth, A.J., Bolton, H., Potter, J., and Priddle, J.D. (1984) in *Methods in Enzymology* (Jacoby, W.B., ed.) Vol. 104, pp. 318–328, Academic Press, Orlando.
12. Laemmli, U.K. (1970) *Nature 227*, 680–685.
13. Heukeshoven, J., and Derrnick, R. (1985) *Electrophoresis 6*, 103–112.
14. Robertson, E.F., Dannelly, H.K., Malloy, P.J., and Reeves, H.C. (1987) *Anal. Biochem. 167*, 290–294.
15. Goullet, P. (1980) *J. Gen. Microbiol. 117*, 483–491.
16. Bradford, M.M. (1976) *Anal. Biochem. 72*, 248–254.
17. Baillargeon, M.W. (1990) *Lipids 25*, 841–848.
18. Haas, M.J., Cichowicz, D.J., and Bailey, D.G. (1992) *Lipids 27*, 571–576.
19. Szatjer, H., Lunsdorf, H., Erdmann, H., Menge, U., and Schmid, R. (1992) *Biochim. Biophys. Acta 1124*, 253–261.
20. Kundu, M., Basu, J., Guchhait, M., and Chakrabarti, P. (1987) *J. Gen. Microbiol. 133*, 149–153.
21. Boel, E., Huge-Jensen, B., Christensen, M., Thim, L., and Fiil, N.P. (1988) *Lipids 23*, 701–706.
22. Verger, R. (1984) in *Lipases* (Borgström, B., and Brockman, H.L., eds.) pp. 83–150, Elsevier, Amsterdam.
23. Tyski, S., Hryniewicz, W., and Jeljaszewicz, J. (1983) *Biochim. Biophys. Acta 749*, 312–317.
24. Borgström, B., and Brockman, H.L. (1984) *Lipases*, Elsevier, Amsterdam.
25. Moreau, R.A. (1987) *J. Dairy. Sci. 70*, 1504–1512.
26. Jensen, R.G. (1983) *Lipids 18*, 650–657.
27. Gargouri, Y., Moreau, H., and Verger, R. (1989) *Biochim. Biophys. Acta 1006*, 255–271.
28. Otero, C., Cruzado, C., and Ballesteros, A. (1991) *Appl. Biochem. Biotechnol. 27*, 185–194.

[Received July 28, 1994, and in revised form February 8, 1995; Revision accepted February 22, 1995]

Induction of Geranyl Pyrophosphate Pyrophosphatase Activity by Cholesterol-Suppressive Isoprenoids

Gary L. Case, Lei He, Huanbiao Mo and Charles E. Elson*

Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT: Diets supplemented (1 mmol/kg) with thymol, carvacrol, and ß-ionone significantly decreased the serum cholesterol levels of cockerels. These mevalonate-derived end products of plant secondary metabolism (isoprenoids) had no impact on two cytosolic prenyl alcohol (and ethanol) dehydrogenase activities; each treatment increased microsomal geranyl pyrophosphate pyrophosphatase activity by greater than twofold. The structural diversity of the isoprenoids which suppress cholesterol synthesis may be reconciled by their ability to increase pyrophosphatase activity, thus leading to the production of the endogenous, post-transcriptional regulator of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity.
Lipids 30, 357–359 (1995).

Mevalonate-derived end products of plant secondary metabolism (isoprenoids) have a cholesterol-lowering effect and a tumor-suppressive action. Both actions may be coupled to the suppression of mevalonate synthesis (1,2). Diverse isoprenoids, including cyclic monoterpenes (3), ß-carotene (4), the tocotrienols (5), farnesyl-pyrophosphate (PP) analogues (6), and farnesol (7), down-regulate microsomal 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase activity by a post-transcriptional action. Mevalonic acid (8) and prenyl-PP containing one (9) to four (7) isoprene units similarly down-regulate HMG–CoA reductase activity. The regulatory actions triggered by the isoprenoids (3–7) reflect those of the physiological, mevalonate-derived nonsterol regulatory agent which acts post-transcriptionally to down-regulate HMG–CoA reductase activity (8).

A microbiological screen (10) of diverse isoprenoids revealed the potent mevalonate-suppressive activities of ß-ionone, carvacrol (2-methyl-5-isopropylphenol), and thymol (2-isopropyl-5-methylphenol) (unreported data). In a follow-up study we found that diets supplemented with 0.5–1.3 mmol/kg ß-ionone, an end-ring analog of ß-carotene, suppressed HMG reductase activities in livers of White Leghorn cockerels and pullets by 45–70% (11).

*To whom correspondence should be addressed at Department of Nutritional Sciences, University of Wisconsin, 1415 Linden Dr., Madison, WI 53706.

Abbreviations: HMG–CoA, 3-hydroxy-3-methylglutaryl CoA; PP, pyrophosphate.

Our experiments draw on reports that cells incubated with saturating concentrations of mevalonic acid accumulate α,ω-prenyl dicarboxylic acids (12). Following the hydrolysis of prenyl-PP intermediates by a microsomal phosphatase (13), cytosolic dehydrogenase and microsomal monooxygenase activities catalyze the formation of prenyl aldehydes, α-prenoic acids, ω- and ω3 hydroxy–α-prenoic acids and α,ω-prenyl dicarboxylic acids (9,12–16). We recently reported that pentobarbital, an inducer of the microsomal P450 monooxygenase activity that catalyzes the formation of ω- and ω3 hydroxy–α-prenoic acids, totally reverses the isoprenoid-mediated suppression of HMG–CoA reductase activity (11). This finding and the postulation by Watson and colleagues that changes in the post isopentenyl-PP isopentenoids individually or in combination define the regulatory signal molecule pool (9,12,14,15) are compatible with the recent report that farnesol induces the degradation of HMG–CoA reductase in permeablized cells (7). Correll *et al.* (7) further report that the induction of protein degradation by farnesyl-PP, the putative regulator identified by Bradfute and Simoni (6), is inhibited by NaF (7). We suggest that the cyclic monoterpenes (3), ß-carotene (4), and the tocotrienols (5) that lower HMG–CoA reductase activity divert a key phosphorylated intermediate of sterol synthesis toward the synthesis of the physiological regulatory agent. These considerations led us to examine the impact of dietary isoprenoids on the reactions preceding the formation of the ω- and ω3 hydroxy–α-prenoic acids.

METHODS

Four groups (n = 8) of White Leghorn cockerels, 126 g, were fed a growing ration (control) or the same ration supplemented with 1 mmol/kg diet of one of the aforementioned isoprenoids [all from Aldrich Chemical Co. (Milwaukee, WI)]. Serum cholesterol levels and three hepatic enzyme activities, microsomal prenyl-PP pyrophosphatase and cytosolic prenyl alcohol and ethanol dehydrogenases were measured following a 21-d feeding period. Body weight gains were not influenced by the treatments. Serum cholesterol levels were measured with diagnostic kits (Sigma Chemical Co., St. Louis, MO). Enzyme activity assays were conducted using concentrations of substrates that were determined experimentally to

Copyright © 1995 by AOCS Press

be saturating. Microsomal prenyl-PP pyrophosphatase activity was determined by measuring the conversion of [1-^{3}H]-geranyl-PP to ^{3}H-geraniol, using a method adapted from those previously described by Christophe and Popják (13) and Croteau and Karp (17). The incubation mixture contained 50 mM Tris (pH 7.4), 100 µM geranyl-PP (Sigma), 0.03 µCi [1-^{3}H]-geranyl-PP (American Radiolabeled Chemicals, Inc., St. Louis, MO) and approximately 400 mg of microsomal protein. The reaction was carried out in a 200 µL volume at 37°C for 20 min. At the end of the incubation period, geraniol was extracted with 1 mL of hexane and counted by liquid scintillation. Results were corrected for small amounts of radioactivity extracted from heat-inactivated tissue blank samples and for geraniol extraction efficiency (90%). Reaction rates were linear with incubation time and amount of tissue extract employed. Prenyl alcohol and ethanol dehydrogenase activities were determined spectrophotometrically at 30°C by following the initial rate of reduction of NAD^+ at 340 nm. The reaction mixture contained 50 mM Tris buffer (pH 7.4), 2 mM NAD^+, 5 mM geraniol, farnesol, or ethanol, and approximately 50 µg of cytosolic protein in a total volume of 1 mL.

RESULTS AND DISCUSSION

Cockerels fed diets supplemented (1 mmol/kg diet) with carvacrol ($P < 0.005$), thymol ($P < 0.05$), or ß-ionone ($P < 0.001$) had a reduced serum cholesterol level as compared to the controls; differences between treatment groups were not significant (Table 1). The isoprenoid-mediated depression of serum cholesterol concentration ranged from 9 to 18%. Under the conditions employed in this study, that is, birds fed an isoprenoid-supplemented, cholesterol-free diet, a 5% suppression of HMG–CoA reductase activity was associated with a 2% decrease in serum cholesterol ($P < 0.004$) (18).

Microsomal geranyl-PP pyrophosphatase activities in livers of birds fed carvacrol ($P < 0.01$), thymol ($P < 0.005$), and ß-ionone ($P < 0.005$) were >2.2-fold greater than the control activity (Table 1). The induction of microsomal prenyl-PP pyrophosphatase activity may represent a key common site of action for the diverse plant isoprenoids that alter cholesterol metabolism in animals. Although this activity diverts prenyl-PPs from the sterologenic pathway, the diversion is not of sufficient magnitude to suppress the incorporation of mevalonate into cholesterol (5,6,19). More likely this activity increases the intracellular concentration of the post isopentenyl-PP metabolite(s) that accelerates the degradation of HMG–CoA reductase (6,7,9,12,14,15). The finding that the farnesol-induced degradation of HMG–CoA reductase, unlike that of farnesyl-PP (7) and farnesyl acetate (6), occurred with no apparent lag time led Correll *et al.* (7) to suggest that farnesol has a more direct role in enzyme degradation.

It should be noted that none of the studies leading to the identification of farnesol as the physiological regulator of HMG–CoA reductase activity (5–7) employed an inhibitor of the prenyl dehydrogenase activity. It remains possible that an inhibitor targeted to the constitutive prenyl alcohol dehydrogenases (Table 1) might potentiate or attenuate the HMG–CoA reductase-suppressive action of farnesol depending on whether farnesol or a farnesol metabolite proves to be the physiological regulator.

Human liver prenyl alcohol dehydrogenase exists in multiple forms with Kms for geraniol and farnesol ranging from 0.2 to 73 and <0.1 to 3 µM, respectively (16). Keung (16) has suggested that genetic determinants of the prenyl alcohol de-

TABLE 1
Effect of Feeding Carvacrol, Thymol, or ß-Ionone on Serum Cholesterol and Hepatic Ethanol and Prenyl Alcohol Dehydrogenase and Geranyl-Pyrophosphate Pyrophosphatase Activities[a]

	Control	Carvacrol	Thymol	ß-Ionone	P[b]
Serum cholesterol (mmol/L)	4.69 ± 0.29	3.85 ± 0.65	4.28 ± 0.35[c]	4.03 ± 0.33[f]	<0.01
Geranyl-PP pyrophosphatase (nmol/min/mg, microsomal protein)	0.124 ± 0.051	0.283 ± 0.073[d]	0.276 ± 0.058[e]	0.270 ± 0.082[e]	<0.005
Geraniol dehydrogenase (nmol/min/mg, cytosolic protein)	17.5 ± 4.7	21.3 ± 5.1	18.3 ± 4.0	15.3 ± 3.7	n.s.
Farnesol dehydrogenase (nmol/min/mg, cytosolic protein)	1.17 ± 0.84	0.87 ± 0.58	0.85 ± 0.48	1.25 ± 0.55	n.s.
Ethanol dehydrogenase (nmol/min/mg, cytosolic protein)	1.24 ± 0.50	1.00 ± 0.46	0.88 ± 0.54	1.05 ± 0.45	n.s.

[a]Cockerels were fed either a growing ration (Control) or the Control ration supplemented with 1 mmol/kg of carvacrol, thymol, or ß-ionone for 21 d; mean ± S.D. n.s., Not significant.
[b]F-test of treatment effects, one-way analysis of variance.
[c–f]Pairwise comparisons with control; [c]$P < 0.05$; [d]$P < 0.01$; [e]$P < 0.005$; [f]$P < 0.001$.

hydrogenase isozymes influence the disposition of products diverted from the sterologenic pathway; differences in prenyl alcohol dehydrogenase isozyme characteristics thus may contribute to differences in serum cholesterol levels among various populations.

This study, to our knowledge, is the first to show the induction of a prenyl-PP pyrophosphatase activity. The disparity between this activity and the prenyl alcohol dehydrogenase activities is consistent with our suggestion that the microsomal prenyl-PP pyrophosphatase reaction is more important in the synthesis of a physiological regulator of HMG–CoA reductase activity.

ACKNOWLEDGMENT

This work was supported in part by UW-CALS project 2656 and USPHS Grant CA 49416.

REFERENCES

1. Elson, C.E. (1995) *J. Nutr.*, in press.
2. Elson, C.E., and Yu, S.G. (1994) *J. Nutr. 124*, 607–614.
3. Clegg, R.J., Middleton, B., Bell, G.D., and White, D.A. (1982) *J. Biol. Chem. 257*, 2294–2299.
4. Moreno, F., Rossiello, M.R., Manjeshwar, S., Nath, R., Rao, P.M., Rajalakshmi, S., and Sarma, D.S.R. (1994) *Proceed. Amer. Assn. Cancer Res. 35*, 142.
5. Parker, R.A., Pearce, B.C., Clark, R.W., Gordon, D.A., and Wright, J.J.K. (1993) *J. Biol. Chem. 268*, 11230–11238.
6. Bradfute, D.L., and Simoni, R.D. (1994) *J. Biol. Chem. 269*, 6645–6650.
7. Correll, G.C., Ng, L., and Edwards, P.A. (1994) *J. Biol. Chem. 269*, 17390–17393.
8. Goldstein, J., and Brown, M.S. (1990) *Nature 343*, 425–430.
9. Giron, M.D., Havel, C.H., and Watson, J.A. (1994) *Proc. Natl. Acad. Sci. USA 91*, 6398–6402.
10. Fitch, M., Mangels, A.R., Altmann, W.A., El Hawary, M., Qureshi, A.A., and Elson, C.E. (1989) *J. Agric. Food Chem. 37*, 687–691.
11. Yu, S.G., Abuirmeileh, N.M., Qureshi, A.A., and Elson, C.E. (1994) *J. Agric. Food Chem. 42*, 1493–1496.
12. Gonzales-Pacanowska, D., Arisan, B., Havel, C.M., and Watson, J.A. (1988) *J. Biol. Chem. 263*, 1301–1306.
13. Christophe, J., and Popják, G. (1961) *J. Lipid Res. 2*, 244–257.
14. Havel, C.M., and Watson, J.A. (1992) *Arch. Biochem. Biophys. 294*, 639–646.
15. Giron, M.D., Havel, C.M., and Watson, J.A. (1993) *Arch. Biochem. Biophys. 302*, 265–271.
16. Keung, W-M. (1991) *Biochem. Biophys. Res. Commun. 174*, 701–707.
17. Croteau, R., and Karp, F. (1979) *Arch. Biochem. Biophys. 198*, 523–532.
18. Elson, C.E., Yu, S.G., and Qureshi, A.A. (1995) *Lipids*, in press.
19. Crowell, P.L., Chang, R.R., Ren, Z., Elson, C.E., and Gould, M.N. (1991) *J. Biol. Chem. 266*, 17679–17685.

[Received July 21, 1994, and in final revised form January 18, 1995; Revision accepted January 23, 1995]

Effects of Pregnenolone-16α-carbonitrile on the Metabolism of Cholesterol in Rat Liver Microsomes

Dagny Ståhlberg*

Department of Medicine, Karolinska Institutet at Huddinge University Hospital, Stockholm, Sweden

ABSTRACT: The effects of pregnenolone-16α-carbonitrile (PCN) on hepatic metabolism of cholesterol were studied in rat liver microsomes in order to clarify the underlying mechanisms of the PCN-induced biliary hypersecretion of cholesterol. Male Sprague-Dawley rats were fed a diet supplemented with 0.05% of PCN for one week. The microsomal activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, regulating cholesterol biosynthesis, decreased from 577 ± 46 (SEM) to 367 ± 38 pmol/min/mg protein compared to the controls. Cholesterol 7α-hydroxylase activity, governing bile acid synthesis, was 9.0 ± 1.1 pmol/min/mg protein in the treated group and 34.8 ± 7.4 pmol/min/mg protein in the controls, a reduction of 74% ($P < 0.01$). The acyl CoA:cholesterol acyltransferase (ACAT) activity, catalyzing the esterification of cholesterol, remained unchanged, as did the levels of total and free cholesterol in liver homogenates and microsomes. The results of this study provide evidence that the increase in biliary cholesterol secretion during PCN treatment is not caused by a change in ACAT activity, but can be explained by a decreased catabolism of cholesterol to bile acids.

Lipids 30, 361–364 (1995).

Pregnenolone-16α-carbonitrile (PCN) is a potent catatoxic steroid which is known to induce hepatic microsomal mixed-function oxygenases, thereby making the liver more resistant to various toxic drugs. Previous studies with rats have shown that PCN also increases the rate of bile secretion and causes bile to be saturated with cholesterol because of an increased cholesterol secretion and an unchanged or decreased output of bile acids and phospholipids (1,2). The enhanced rate of bile flow could be explained by increased bile salt-independent bile flow through induction of the activity of canalicular membrane Na^+K^+-dependent adenosine triphosphatase (1–3). The mechanism of the enhanced biliary secretion of cholesterol during PCN treatment has not been fully explained, but it may be due to effects of PCN on the hepatic metabolism of cholesterol—increased hepatic synthesis, decreased catabolism to bile acids and reduced cholesteryl ester formation. Thus, the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme for bile acid formation, has been shown to decrease slightly in rats treated with PCN (4,5).

Nervi *et al.* (6) and Stone *et al.* (7) have previously studied the interrelationship between hepatic cholesterol esterification and biliary cholesterol output in rats, and they demonstrated that an inhibition of the acyl CoA:cholesterol acyltransferase (ACAT) activity was associated with increased biliary cholesterol secretion. The effect of PCN on ACAT activity has not previously been studied, but Turley and Dietschy (2) found an increase in cholesteryl esters when female rats were fed a diet containing PCN.

The present work aimed at studying further the mechanism of the previously reported PCN-induced hypersecretion of cholesterol in rats. The activities of 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase, cholesterol 7α-hydroxylase, and ACAT, the three key enzymes in the regulation of the hepatic cholesterol synthesis and metabolism, were simultaneously determined in male Sprague-Dawley rats treated with 0.05% PCN added to the food. In addition, the concentrations of free and esterified cholesterol in liver homogenates and microsomes were measured.

MATERIALS AND METHODS

Materials. Deuterium-labeled cholesterol was obtained from Larodan Fine Chemicals (Malmö, Sweden). Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (8). [3-^{14}C]HMG–CoA (specific activity 19 mCi/mmol), [1-^{14}C]oleoyl coenzyme A (specific activity 57.8 mCi/mmol) and [1,2,6,7-^{3}H]cholesteryl oleate (specific activity 82.7 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). DL-[2-^{3}H]mevalonic acid (specific activity 125 mCi/mmol) was obtained from Radiochemical Centre (Amersham, England). Unlabeled mevalonic acid lactone, cholesteryl oleate, NADP, NADPH, EDTA, dithiothreitol (DTT), glucose-6-phosphatate, glucose-6-phosphate dehydrogenase, and human serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). PCN was obtained from Upjohn Co. (Kalamazoo, MI).

*Address correspondence at Dept. of Medicine, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; DTT, dithiothreitol; HMG–CoA, 3-hydroxy-3-methylglutaryl CoA; PCN, pregnenolone-16α-carbonitrile.

Copyright © 1995 by AOCS Press

Experimental design. Two groups of six male rats of the Sprague-Dawley strain, weighing about 170 g, were used. Both groups had free access to water and a crushed commercial standard diet. In one of the groups the food was supplemented with 0.05% (w/w) of PCN. The other group served as controls. After one week of treatment, the animals in both groups were killed, the livers were immediately removed, weighed, cut into two pieces and put in cold buffer solutions. The weight of the rats was recorded immediately before the initial feeding of the experimental diet and again on the day of the experiment.

Preparation of liver microsomes. An aliquot of the liver was minced and homogenized with a loose-fitting Teflon pestle in nine volumes of a 50 mM TRIS–HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM DTT, 10 mM EDTA, and 50 mM NaCl. The homogenate was centrifuged at 20,000 × *g* for 15 min. The supernatant solution was centrifuged at 100,000 × *g* for 60 min. Half of the microsomal fraction prepared was resuspended in the homogenizing medium and recentrifuged at 100,000 × *g* for 60 min. This fraction was then suspended in a 20 mM imidazole buffer, pH 7.4, containing 10 mM DTT in a volume corresponding to that of the 20,000 × *g* supernatant, and used for the assay of HMG–CoA reductase. Half of the original microsomal fraction was recentrifuged at 100,000 × *g* in a homogenizing medium lacking DTT. The resulting fraction was suspended in a 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and used for the assay of cholesterol 7α-hydroxylase activity.

The other piece of the liver was minced and homogenized in 50 mM TRIS–HCl buffer, pH 7.4, containing 0.3 M sucrose and 1 mM EDTA, as described above. The homogenate was centrifuged at 20,000 × *g* for 15 min. The supernatant solution was centrifuged at 100,000 × *g* for 60 min. The pellet obtained was resuspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, and used for assay of the ACAT activity.

The microsomal and the liver homogenate contents of protein were determined by the method of Lowry *et al.* (9).

Assay of HMG–CoA reductase activity. The HMG–CoA reductase activity was assayed essentially as described (10). The microsomal fraction, 40 μL, was preincubated for 15 min at 37°C in a total volume of 0.2 mL containing 100 mM phosphate buffer, pH 7.4, 10 mM imidazole buffer, pH 7.4, 5 mM DTT, 10 mM EDTA, 3 mM NADP, 12 mM glucose-6-phosphatase, and 1 unit of glucose-6-phosphate dehydrogenase. The HMG–CoA reductase assay was then initiated with the addition of 90 nmol of [3-^{14}C]HMG–CoA (0.5 μCi) dissolved in 25 μL of distilled water. The incubation was run for 15 min and was stopped by the addition of 25 μL of 6 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) was added as an internal standard together with 3 mg of unlabeled mevalonic acid lactone, and the incubation mixture was allowed to stand overnight at room temperature for complete lactonization. The incubation mixture was then subjected to thin-layer chromatography using toluene/acetone (1:1, vol/vol) as the developing solvent. The zone containing mevalonic acid lactone was visualized with iodine vapor and scraped off, and the radioactivity was counted. Corrections for losses were made based on the internal standard.

Assay of cholesterol 7α-hydroxylase activity. The standard assay system consisted of 1.0 mL of the microsomal fraction, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM NADPH. The enzyme assay was carried out in duplicate for 15 min at 37°C according to the method of Björkhem and Kallner (8) where the product 7α-hydroxycholesterol was analyzed by gas chromatography/mass spectrometry. Each set of experiments also included two assays in which the mass of 7α-hydroxycholesterol was determined in the incubation mixture extracted at zero time. The amount of 7α-hydroxycholesterol formed during an incubation was estimated by subtracting the amount present at zero time from the amount present at the end of the incubation period and was expressed as pmol/min/mg protein.

Assay of ACAT enzyme activity. The ACAT activity was assayed both in the absence and presence of exogenous cholesterol as described previously (11). The assay system contained 0.1 mL of the microsomal preparation and 1 mg of fatty acid free bovine serum albumin in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, to give a final volume of 1.0 mL. The mixture was preincubated for 5 min at 37°C or for 30 min at 37°C after the addition of 50 nmol of unlabeled cholesterol dissolved in 600 μg of Triton. The reaction was initiated by addition of 25 nmol (1.45 μCi) of [1-^{14}C]oleoyl CoA. After 4 min the assay was stopped by the addition of 10 mL chloroform/methanol (2:1, vol/vol). Tritium-labeled cholesteryl oleate (0.01 μCi) was added as an internal standard to estimate recovery followed by 1 mL 0.9% (wt/vol) NaCl.The chloroform phase was collected and evaporated to dryness under N_2. The residue was resuspended in chloroform/methanol (2:1, vol/vol) and subjected to thin-layer chromatography together with unlabeled cholesteryl oleate as marker. The chromatogram was developed in hexane/ethyl acetate (95:5, vol/vol). The cholesteryl oleate zone was visualized with iodine vapor, scraped into a vial, and counted.

Determination of liver cholesterol. The concentrations of total cholesterol in the liver homogenates and the microsomal fractions were determined by isotope dilution–mass spectrometry after addition of deuterium-labeled cholesterol as an internal standard as described previously (12) with modifications (13). Free cholesterol was determined by the same method, but without the hydrolysis step. The concentration of esterified cholesterol was calculated as the difference between total and free cholesterol in the same sample.

Statistical analysis. Data are presented as means ± SEM (standard error of the mean). The significance of differences was evaluated by Student's *t*-test.

RESULTS

The PCN-treated rats consumed the same quantity of food as the control group and tolerated the treatment well. The weight gain of the rats during the treatment period with PCN did not

TABLE 1
Effects of Pregnenolone-16α-Carbonitrile (PCN) Treatment on Body and Liver Weight

	Number of rats	Weight gain (g)	Liver weight (g)
Controls	6	52 ± 2	10.5 ± 0.3
PCN treatment	6	52 ± 3	13.2 ± 0.5[a]

[a]Significantly different from the controls; $P < 0.001$.

differ from that of the controls. Treatment with PCN significantly increased the liver weight by 25% (Table 1). The protein content in the liver microsomes increased slightly in the treated rats as compared to the controls (1.97 ± 0.13 and 1.65 ± 0.20 mg/mL, respectively), but the difference did not reach statistical significance. The protein content in liver homogenate was about the same in the treated group and in the controls.

The activity of HMG–CoA reductase was about 35% lower in the group of rats treated with PCN compared to the controls (367 ± 38 and 577 ± 46 pmol/min/mg protein, respectively) (Table 2). The activity of cholesterol 7α-hydroxylase was 34.8 ± 7.4 pmol/min/mg protein in the controls and 9.0 ± 1.1 pmol/min/mg protein in the treated group, a decrease of nearly 75%. The ACAT activity, determined without exogenous cholesterol added, remained unchanged in the rats treated with PCN. When exogenous cholesterol was added to the assay system, the ACAT activity increased sevenfold in the controls, as well as in the PCN-treated rats (Table 2). The concentrations of free and esterified cholesterol in the liver homogenates and the microsomes were not significantly changed in the PCN-treated rats (Table 3).

DISCUSSION

The effects of PCN on the three key enzymes, regulating the hepatic cholesterol metabolism, were studied in male Sprague-Dawley rats. From previous studies it is known that PCN stimulates the rate of bile secretion and increases biliary cholesterol output (1–3). In the present study, when the rats were fed a diet containing 0.05% PCN, the weight of the liver increased by 25%, a well-known effect of PCN treatment (1–3,5), and explained by a proliferation of the smooth-surfaced endoplasmic reticulum in the hepatocytes (4,14). In the present study, the concentration of microsomal protein showed a tendency to increase.

The activity of HMG–CoA reductase, regulating the synthesis of cholesterol in the liver, decreased by about 35%, and the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid formation, was reduced by about 75%. Honohan and Parkinson (5) found an increased total cholesterol turnover, as well as a slight inhibition of cholesterol 7α-hydroxylase, in rats treated with PCN.

The activity of ACAT, determined both without and with exogenous cholesterol added, was not affected by PCN treatment, and, in accordance with this, the levels of cholesteryl esters in liver homogenates and microsomes remained unchanged compared to the control group. In contrast, Turley and Dietschy (2) found an increase in the amount of liver cholesteryl esters when female Sprague-Dawley rats were treated with PCN at the same dietary level as that used in this study. There is no apparent explanation of the different results obtained in the present study and in that of Turley and Dietschy (2). But one contributing factor might be that different sexes of rats were used (15).

TABLE 2
The Microsomal Enzyme Activities (pmol/min/mg protein) in Controls and in PCN -Treated Rats[a]

			ACAT	
	HMG–CoA reductase	Cholesterol 7α-hydroxylase	Endogenous cholesterol	+ Exogenous cholesterol
Controls	577 ± 46	34.8 ± 7.4	371 ± 24	2582 ± 327
PCN-treated	367 ± 38[b]	9.0 ± 1.1[b]	352 ± 30	2464 ± 368

[a]Abbreviations as in Table 1; HMG–CoA, 3-hydroxy-3-methylglutaryl CoA; ACAT, acyl CoA:cholesterol acyltransferase.
[b]Significantly different from' the controls; $P < 0.01$.

TABLE 3
The Concentrations of Total and Esterified Cholesterol (nmol/mg protein) in Liver Homogenates and Microsomes in Controls and in PCN-Treated Rats[a]

	Liver homogenates		Microsomes	
	Total cholesterol	Esterified cholesterol	Total cholesterol	Esterified cholesterol
Controls	34.4 ± 2.2	6.2 ± 1.2	55.5 ± 8.2	8.5 ± 2.3
PCN-treated	35.6 ± 4.2	7.8 ± 1.8	51.3 ± 5.1	9.1 ± 1.0

[a]Values are means ± SEM of six determinations. None of the differences were statistically significant. Abbreviation as in Table 1.

The liver is the main organ through which cholesterol is cleared from the body either unchanged or after conversion to bile acids (16). An imbalance between the excretion of cholesterol and bile acids may cause an excess of cholesterol in bile, as has been reported for PCN-treated rats. Such an imbalance may theoretically be due to one of several factors, e.g., an enhanced synthesis of cholesterol (HMG–CoA reductase activity) in the liver; a decreased catabolism of cholesterol to bile acids as reflected in a decreased activity of cholesterol 7α-hydroxylase; a reduced esterification rate of cholesterol (ACAT activity) in the liver, leading to a decreased storage of cholesterol in the liver as cholesteryl esters. We did not find that PCN affected the esterification rate or storage of cholesterol in the liver; nor did we find that PCN stimulated the HMG–CoA reductase activity; rather the HMG–CoA reductase activity was reduced. The most pronounced effect of PCN was a marked decrease in the conversion of cholesterol to 7α-hydroxycholesterol. Decreased cholesterol 7α-hydroxylase activity in PCN-treated rats has previously been demonstrated by Einarsson and Gustafsson (4) and Honohan and Parkinson (5).

The results of this study, together with evidence from other laboratories, support the conclusion that the increased biliary cholesterol output during PCN treatment of rats is not regulated by the rate of cholesterol esterification, but may be related to the pronounced decrease in the rate of cholesterol 7α-hydroxylation.

ACKNOWLEDGMENTS

The skillful technical assistance of Gunvor Alvelius, Ingela Arvidsson and Lisbet Benthin is gratefully acknowledged. This study was supported by grants from the Swedish Medical Research Council (03X-04793) and from the Karolinska Institutet.

REFERENCES

1. von Bergmann, K., Schwarz, H.P., and Paumgartner, G. (1975) *Naunyn-Schmiedeberg's Arch. Pharmacol. 287,* 33–45.
2. Turley, S.D., and Dietschy, D.M. (1984) *Gastroenterology 87,* 284–292.
3. Zsigmond, G., and Solymoss, B. (1974) *Proc. Soc. Exp. Biol. Med. 145,* 631–635.
4. Einarsson, K., and Gustavsson, J-Å. (1973) *Eur. J. Biochem. 32,*197–206.
5. Honohan, T., and Parkinson, T.M. (1975) *Biochem. Pharmacol. 24,* 899–903.
6. Nervi, F., Bronfman, M., Allalon, W., Depiereux, E., and Del Pozo, R. (1984) *J. Clin. Invest. 74,* 2226–2237.
7. Stone, B.G., Erickson, S.K., Craig, W.Y., and Cooper, A.D. (1985) *J. Clin. Invest. 76,* 1773–1781.
8. Björkhem, I., and Kallner, A. (1976) *J. Lipid Res. 17,* 360–365.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
10. Angelin, B., Einarsson, K., Liljeqvist, L., Nilsell, K., and Heller, R.A. (1984) *J. Lipid Res. 25,* 1159–1166.
11. Ståhlberg, D., Angelin, B., and Einarsson, K. (1989) *J. Lipid Res. 30,* 953–958.
12. Björkhem, I., Blomstrand, R., and Svensson, L. (1974) *Clin. Chim. Acta 54,*185–193.
13. Schaffer, R., Sniegoski, L.T., Welch, M.J., White, E., Cohen, V.A., Hertz, H.E., Mandel, J., Paule, T.C., Svensson, L., Björkhem, I., and Blomstrand, R. (1982) *Clin. Chem. 28,* 5–8.
14. Garg, B.D., Kovacs, K., Blascheck, J.A., and Selye, H. (1970) J. *Pharm. Pharmacol. 22,* 872–873.
15. Zsigmond, G., and Solymoss, B. (1972) *J. Pharmacol. Exp. Ther. 183,* 499–507.
16. Turley, S.D., and Dietschy, J.M. (1988) in *The Liver: Biology and Pathology,* 2nd edn. (Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D., and Shafritz, D.A., eds.) pp. 617–641, Raven Press, New York.

[Received May 21, 1994, and in revised form January 23, 1995; Revision accepted January 25, 1995]

Fatty Acid Composition of Erythrocyte, Platelet, and Serum Lipids in Strict Vegans

Jyrki J. Ågren*, Marja-Leena Törmälä, Mikko T. Nenonen and Osmo O. Hänninen

Department of Physiology, University of Kuopio, 70211 Kuopio, Finland

ABSTRACT: The fatty acid composition of erythrocytes, platelets, and serum lipids was compared between subjects who had been eating a strict uncooked vegan diet ("living food") for years and omnivore controls. The vegan diet contains equal amounts of fat but more monounsaturated and polyunsaturated and less saturated fatty acids than the mixed diet of the control group. In vegans, the proportion of linoleic acid was greater in all lipid fractions studied. Also, the levels of other n-6 fatty acids were greater, with the exception of arachidonic acid levels, which were similar in most fractions. In erythrocytes, platelets and serum phospholipid fractions, this increase was mainly at the expense of the n-3 fatty acids. The proportions of eicosapentaenoic and docosahexaenoic acid were only 29–36% and 49–52% of those in controls, respectively. In vegans the ratio of n-3 to n-6 fatty acids was only about half that in omnivores. In addition to the lower levels of n-3 fatty acids, the proportions of palmitic and stearic acids were lower in serum cholesteryl esters, triglycerides and free fatty acids of vegans. The proportion of oleic acid was slightly lower only in serum cholesteryl esters and erythrocyte phosphatidylserine. The results show that, in the long term, the vegan diet has little effect on the proportions of oleic and arachidonic acids, whereas the levels of n-3 fatty acids are depressed to very low levels with prolonged consumption of the high linoleic and oleic acid components of this diet. *Lipids 30,* 365–369 (1995).

In numerous studies the fatty acid composition of different blood lipid components has been measured to assess the quality of dietary fat or to clarify the effects of dietary fat modification or fatty acid supplementation. It is well known that the amounts of several fatty acids in blood lipids reflect, to some extent, their dietary intake (1–4). However, there is little firm information about the long-term effects of different dietary combinations of saturated, monounsaturated and n-6 and n-3 fatty acids on plasma and membrane lipids. In addition, methodological problems may complicate the interpretation of some published data.

One possible way to elucidate the relations between dietary fatty acid intake and the fatty acid composition of blood lipids is to study the subjects, such as vegetarians, who follow a special lifestyle diet. Vegetarian diets are known to have positive effects on weight, blood pressure and coronary artery disease (5). It should be noted that in addition to the nutrient content of their diet, other aspects of the lifestyles of vegans may contribute to these positive health effects. The fatty acid composition is one feature of the diet which is clearly different in vegetarian and omnivore diets. The intake of linoleic acid is high in many vegetarian diets, and this has been clearly shown to increase its proportion in platelet phospholipids (PL) (6) and in plasma phosphatidylcholine (PC), erythrocytes and adipose tissue (7), when vegans have been compared to omnivore controls. The only significant difference in the major fatty acids of serum lipids was the lower level of arachidonic acid in cholesterol esters and phospholipids of vegans in the study of Phinney *et al.* (8). Melchert *et al.* (9) found only small differences in arachidonic acid concentrations, but the proportion of linoleic acid was greater, whereas the proportions of oleic and docosahexaenoic acids were lower in vegans than in omnivores. Unfortunately, dietary data were included in one only of these reports (6).

In the present study, fatty acids of blood lipids were analyzed from subjects who had consumed a vegan diet ("living food") for many years. "Living food" is a strict uncooked vegan diet, rich in lactobacilli, which contains no animal products or added salt. The amounts of cholesterol and saturated fatty acids are very low in this diet. Monounsaturated fatty acids constitute about half and polyunsaturated fatty acids, mainly linoleic acid, almost one-third of the fatty acids available in the diet (10). The subjects have participated in a special course where the preparation of the diet has been taught and the recommended weekly schedule learned.

EXPERIMENTAL PROCEDURES

Originally twelve "living food" consumers and twelve control subjects with a normal mixed diet volunteered to participate in this study. Two subjects of the vegan group were found not to be following strictly to the diet. The rest were requested to come to give a blood sample for fatty acid analyses. Blood samples were obtained from nine vegans and eleven controls. One subject of the vegan group was excluded from the final results due to very exceptional fatty acid pro-

*To whom correspondence should be addressed at Department of Physiology, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland.

Abbreviations: CE, cholesteryl esters; FFA, free fatty acids; PC, phosphatidylcholine; PL, phospholipids; PS, phosphatidylserine; TG, triglycerides; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

TABLE 1
Anthropometric and Dietary Summaries in Strict Vegan (living food) and Control Groups

	Controls	Vegans
Number of subjects	11	8
Male/female (ratio)	1:10	1:7
Age (yr)	51 ± 9	47 ± 12
Weight (kg)	62 ± 10	54 ± 7
Height (cm)	165 ± 8	166 ± 8
Body mass index (kg/m^2)	23.8 ± 4.0	19.5 ± 1.2[b]
Energy intake (kJ/d)[c]	6449 ± 1780	7008 ± 2101
Protein (g/d)	58.9 ± 11.6	49.5 ± 12.8
Carbohydrate (g/d)	166.1 ± 55.8	204.7 ± 68.4
Fat (g/d)	63.8 ± 18.0	70.3 ± 32.2
Saturated fatty acids (g/d)	26.5 ± 7.0	9.6 ± 6.5[b]
Monounsaturated fatty acids (g/d)	20.6 ± 7.7	29.8 ± 19.9
Polyunsaturated fatty acids (g/d)	10.4 ± 4.7	18.1 ± 9.0
Linoleic acid (g/d)	6.6 ± 3.2	15.0 ± 7.8[a]
Linolenic acid (g/d)	1.2 ± 0.6	2.9 ± 2.5
Eicosapentaenoic acid (g/d)[d]	0.09 ± 0.05	
Docosahexaenoic acid (g/d)[d]	0.22 ± 0.14	
Cholesterol (mg/d)	235 ± 129	10 ± 23[b]

[a] $P < 0.05$.
[b] $P < 0.001$, Mann-Whitney U-test.
[c] Dietary records were obtained from seven subjects in control group.
[d] Estimated on the basis of fish consumption questionnaire.

files: the proportions of several fatty acids differed by 4–5 standard deviations from the mean values. The vegans included had followed the "living food" diet for at least two years (mean time 7.3 yr).

Subjects kept food records for seven days using household measures. About six months elapsed between the blood sampling and recording the food intake. Therefore they were asked to complete another questionnaire to clarify if any changes, especially in fat intake, had occurred during this period. No changes were observed, emphasizing the stability of their diet. In addition, subjects of the control group filled in a separate questionnaire concerning their consumption of fish. This showed a frequency of 2.0 ± 0.9 fish meals per week (mean ± SD), providing about 0.4 g of n-3 fatty acids per day. Nutrients were analyzed using the software system developed at the Finnish Social Insurance Institute (Helsinki, Finland). The food and nutrition database currently comprises about 1400 food items and 200 nutrients. The composition of some special food items of the living food diet was analyzed and added to the database. The anthropometric data and the intake of basic nutrients, fatty acids and cholesterol are presented in Table 1. The database contains information of total linolenic acid. Therefore, linolenic acid is used instead of α-linolenic acid when dietary intake data is discussed.

The fasting blood samples were collected, and erythrocytes, platelets, and plasma separated as described earlier (11). Lipids from erythrocyte membranes, platelets, and serum samples were extracted by the method of Folch *et al.* (12). Erythrocyte phospholipids were separated by thin-layer chromatography (TLC) (Empore™ TLC; 3M, St. Paul, MN). Serum lipids were separated in a single aminopropyl solid-phase glass column (13). Fatty acid methyl esters were prepared with 14% BF_3–methanol and were analyzed with a Carlo Erba Vega 6130 gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a 25 m × 0.32 i.d. fused silica capillary column (NB-351; HNU-Nordion Ltd., Helsinki, Finland).

The Mann-Whitney U-test was used to statistically compare the differences between the groups.

RESULTS

The anthropometric data in Table 1 shows that vegans are leaner than control subjects despite their equal energy intake. One possible reason for this is that all the nutrients present in this uncooked diet are not utilized as efficiently as in the normal diet. There could also be some underreporting of energy intakes in controls, especially in two subjects with reported energy intakes below 5 MJ/d. However, the fatty acid distribution of controls was very close to the mean distribution for Finnish women. There was no significant difference in total fat intake between the groups, but the vegan diet contained considerably less saturated and more monounsaturated and polyunsaturated fatty acids. The sources of dietary fat were quite different in these groups. Vegans do not use milk products or meat, and the fat is derived mostly from seeds, nuts and avocado. The cholesterol intake of controls was much higher than that of vegans. The small amount of cholesterol found in the vegan diet originated predominantly from egg containing pastry eaten by one subject.

The differences between vegans and their controls in the fatty acid composition of erythrocyte and platelet lipids (Table 2) and serum phospholipids (Table 3) were very similar. The proportions of all n-6 fatty acids were greater in vegans, with the exception of the unchanged levels of arachidonic acid found in most fractions. Its proportion was significantly greater only in erythrocyte phosphatidylserine (PS) of vegans. The greatest difference between the groups was seen in the amount of linoleic acid, but, in relative terms, even greater differences were observed in the minor fractions of long-chain n-6 fatty acids.

The proportion of n-3 fatty acids was much lower in the serum lipids and cell membranes obtained from the vegans. In erythrocytes, platelets, and serum cholesteryl esters (CE) and PL the proportions of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) were only 29–36 and 49–52%, respectively, of those of controls. The proportions of docosapentaenoic acid (22:5n-3) were only slightly lower in vegans. The ratio of n-3 to n-6 fatty acids in vegans was about half of that in omnivores. Only small differences were found in the amounts of saturated and monounsaturated fatty acids in erythrocytes and platelets, as well as in serum PL. The proportion of nervonic acid (24:1n-9) was greater in total erythrocyte lipids of vegans. In erythrocyte PC there was more stearic acid and less palmitic acid in vegans than in the control group. In serum PL the proportion of palmitic acid was also slightly lower in vegans.

TABLE 2
The Fatty Acid Composition of Erythrocyte Membrane Total, Phosphatidylethanolamine (PE), Phosphatidylcholine (PC) and Phosphatidylserine (PS), and Platelet Lipids in Vegans (n = 8) and Controls (n = 11) (mol%, mean ± SD)

	Erythrocyte total		Erythrocyte PE		Erythrocyte PC		Erythrocyte PS		Platelets	
	Controls	Vegans	Controls	Vegans	Controls	Vegans	Controls	Vegans	Controls	Vegans
14:0	0.5 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.6 ± 0.1	0.4 ± 0.1			0.7 ± 0.3	0.6 ± 0.1
16:0	23.0 ± 1.1	23.2 ± 0.8	15.7 ± 1.2	16.3 ± 1.1	34.7 ± 1.5	31.9 ± 2.0^b	3.5 ± 0.9	3.3 ± 0.8	17.9 ± 0.8	17.3 ± 0.9
18:0	15.0 ± 0.6	14.8 ± 0.9	8.1 ± 1.0	7.6 ± 1.1	10.0 ± 1.1	11.3 ± 1.1^a	39.4 ± 1.2	38.6 ± 2.1	18.2 ± 0.7	18.3 ± 0.7
20:0	0.3 ± 0.1	0.3 ± 0.0					0.2 ± 0.0	0.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.2
22:0	1.0 ± 0.1	0.9 ± 0.1							1.6 ± 0.2	1.7 ± 0.3
24:0	2.2 ± 0.3	2.3 ± 0.3							0.8 ± 0.1	1.0 ± 0.2
16:1n-(7 + 9)	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0.6 ± 0.4			0.9 ± 0.2	0.7 ± 0.1
18:1n-9	14.0 ± 0.6	13.3 ± 1.3	16.8 ± 1.3	17.9 ± 1.7	16.3 ± 0.4	14.8 ± 2.2	6.6 ± 0.7	5.5 ± 0.5^c	16.4 ± 0.9	16.0 ± 1.2
18:1n-7	1.5 ± 0.2	1.4 ± 0.2	1.1 ± 0.2	1.2 ± 0.3	1.9 ± 0.2	1.8 ± 0.4	1.3 ± 0.3	0.9 ± 0.2^b	1.7 ± 0.2	1.4 ± 0.2
20:1n-(9 + 11)	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
24:1n-9	2.2 ± 0.3	2.7 ± 0.4^b							1.1 ± 0.1	1.4 ± 0.3
18:2n-6	11.6 ± 1.1	14.2 ± 1.3^b	6.6 ± 1.2	10.0 ± 1.7^c	22.6 ± 2.0	25.4 ± 1.9^a	2.2 ± 0.2	3.3 ± 0.8^b	7.3 ± 0.9	9.5 ± 0.8^c
18:3n-3	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1^a				
20:3n-6	1.5 ± 0.2	2.0 ± 0.5^b	1.3 ± 0.3	2.1 ± 0.6^b	2.0 ± 0.5	2.6 ± 0.5^a	1.9 ± 0.4	3.0 ± 0.8^b	1.7 ± 0.4	2.1 ± 0.5
20:4n-6	13.7 ± 1.0	14.3 ± 1.3	23.8 ± 1.8	24.1 ± 2.1	5.8 ± 1.2	7.5 ± 3.0	23.1 ± 1.7	28.5 ± 2.4^c	22.8 ± 1.4	22.7 ± 1.1^c
20:5n-3	1.4 ± 0.4	0.5 ± 0.2^c	2.9 ± 0.9	1.1 ± 0.6^c	1.1 ± 0.4	0.4 ± 0.2^c	0.9 ± 0.2	0.4 ± 0.2^b	0.9 ± 0.3	0.3 ± 0.2^b
22:4n-6	2.0 ± 0.3	3.0 ± 0.6^c	4.7 ± 0.8	7.0 ± 1.2^c	0.2 ± 0.1	0.5 ± 0.2^a	2.1 ± 0.5	3.6 ± 1.2^b	1.6 ± 0.2	2.1 ± 0.4^b
22:5n-6	0.2 ± 0.1	0.5 ± 0.2^b	0.4 ± 0.1	0.8 ± 0.2^c			0.6 ± 0.1	1.2 ± 0.4^c		
22:5n-3	2.5 ± 0.2	2.1 ± 0.3^a	5.1 ± 0.5	4.3 ± 0.6^b	0.6 ± 0.1	0.7 ± 0.3	4.0 ± 0.6	3.4 ± 0.6	1.7 ± 0.2	1.3 ± 0.4
22:6n-3	6.7 ± 0.9	3.3 ± 1.6^c	12.6 ± 1.6	6.5 ± 3.0^c	3.0 ± 0.6	1.6 ± 1.5^c	14.1 ± 1.4	7.8 ± 3.8^c	2.7 ± 0.5	1.4 ± 0.7^c
n-3	10.7 ± 1.4	6.0 ± 1.7^b	20.8 ± 2.6	12.1 ± 3.6^c	5.0 ± 1.1	2.9 ± 0.7^b	19.0 ± 1.9	11.6 ± 3.8^c	5.3 ± 0.9	3.0 ± 0.9^c
n-6	29.0 ± 1.7	34.0 ± 3.0^b	36.8 ± 2.0	44.0 ± 4.5^c	30.6 ± 1.4	36.0 ± 3.4^b	29.8 ± 1.8	39.6 ± 4.4^c	33.4 ± 2.0	36.4 ± 1.5^b
n-3/n-6	0.37 ± 0.06	0.18 ± 0.06^c	0.57 ± 0.09	0.28 ± 0.11^c	0.17 ± 0.04	0.08 ± 0.02^c	0.64 ± 0.10	0.30 ± 0.13^c	0.16 ± 0.03	0.08 ± 0.03^c

$^a P < 0.05$. $^b P < 0.01$. $^c P < 0.001$.

The serum PL fraction followed the same pattern as erythrocyte and platelet lipids. In the other fractions, the proportion of linoleic acid was also much greater in vegans than in controls [60.6 ± 3.8 compared to 53.7 ± 4.6% for CE, 27.5 ± 5.5 compared to 17.2 ± 5.2% for triglycerides (TG) and 22.4 ± 5.1 compared to 12.6 ± 2.7% for free fatty acids (FFA)] (Table 3). The amounts of n-3 fatty acids were lower in vegans. The greatest difference was seen in serum TG, where the ratio of n-3 to n-6 fatty acids was 0.23 in omnivores and only 0.07 in vegans. In addition to differences in the polyunsaturated fatty acids, the proportions of palmitic and stearic acids were much lower in serum TG (19.4 ± 2.4 compared to 25.0 ± 4.5% and 2.7 ± 0.8 compared to 3.8 ± 1.0%, respectively), and in FFA (22.9 ± 2.3 compared to 29.0 ± 1.9% and 11.9 ± 0.4 compared to 16.2 ± 4.6%, respectively), and also slightly lower in serum CE of vegans. The only significant difference in monounsaturated fatty acids was the lower level of oleic acid in CE of vegans (15.9 ± 2.2 compared to 18.3 ± 2.0%).

The total amount of fatty acids in serum CE, TG and PL of vegans were lower (15–36%) than in the control group, but the differences were not statistically significant. The total amounts of serum FFA were similar in both groups (0.38 mmol/L).

DISCUSSION

The high linoleic acid intake of vegans was reflected in all the lipid fractions studied. Similar findings have also been reported in other studies with vegans and vegetarians (6,7,9), with one exception (8). The effects of the vegetarian diet on other fatty acids have been much more variable. The short-term increase of linoleic acid intake has been shown to increase its amount in serum CE and erythrocyte membranes at the expense of oleic acid (14). In the present study, the proportion of oleic acid was lower only in serum CE of vegans. In the other fractions, the increased n-6 fatty acid content was compensated mainly *via* lowered amounts of n-3 fatty acids in membranes and saturated fatty acids in serum TG and FFA. It seems that the high intake of oleic acid combined with the very low intake of long-chain n-3 fatty acids in vegans leads to the substitution of n-3 fatty acids by n-6 fatty acids instead of oleic acid, especially in PL. Similar trends have also been observed in earlier studies (6,7,9). It seems that with vegetarian diets, which have a higher linoleic/oleic acid ratio than that of the present study, oleic acid is more easily replaced by linoleic acid. However, limited dietary data makes the comparison difficult.

The proportions of n-3 fatty acids vary greatly in different membranes and PL, and these characteristics partly determine the possible extent to which they can be modified by diet. The relative differences between the groups were, however, very similar in erythrocytes, platelets, and serum PL where in vegans the ratio of n-3 to n-6 fatty acids was about half of that in omnivores. In serum cholesterol esters of vegans, this ratio was 40% and in TG 30% of that in omnivores. Thus, the very low intake of long-chain n-3 fatty acids in vegans was most clearly seen in these fractions which are more dependent on daily dietary supplementation than membrane PL. The selec-

TABLE 3
The Fatty Acid Composition of Serum Cholesterol Esters (CE), Triglycerides (TG), Free Fatty Acids (FFA) and Phospholipids (PL) in Vegans (n = 8) and Controls (n = 11) (mol%, mean ± SD)

	CE		TG		FFA		PL	
	Controls	Vegans	Controls	Vegans	Controls	Vegans	Controls	Vegans
14:0	1.1 ± 0.4	0.9 ± 0.4	2.6 ± 1.0	2.2 ± 0.5	4.2 ± 0.7	3.3 ± 0.9[a]	0.5 ± 0.1	0.5 ± 0.1
16:0	11.5 ± 0.7	10.4 ± 1.1[a]	25.0 ± 4.5	19.4 ± 2.4[b]	29.0 ± 1.9	22.9 ± 2.3[c]	30.3 ± 1.7	28.4 ± 1.4[a]
18:0	1.0 ± 0.2	0.7 ± 0.2[a]	3.8 ± 1.0	2.7 ± 0.8[a]	16.2 ± 4.6	11.9 ± 0.4[a]	12.0 ± 1.2	12.3 ± 1.4
20:0							0.4 ± 0.1	0.4 ± 0.1
22:0							0.9 ± 0.3	0.9 ± 0.1
24:0							0.7 ± 0.2	0.8 ± 0.1
16:1n-(7 + 9)	3.8 ± 1.7	2.6 ± 0.9	5.0 ± 1.4	4.8 ± 1.0	4.9 ± 1.4	3.9 ± 0.8	0.8 ± 0.3	0.8 ± 0.3
18:1n-9	18.3 ± 2.0	15.9 ± 2.2[a]	37.2 ± 2.7	36.4 ± 6.0	27.1 ± 4.0	30.0 ± 3.7	9.4 ± 0.8	9.6 ± 1.5
18:1n-7	1.2 ± 0.2	1.0 ± 0.3	2.7 ± 0.6	2.3 ± 0.4	2.6 ± 0.3	2.6 ± 0.3	1.9 ± 0.2	1.9 ± 0.5
24:1n-9							1.9 ± 0.4	1.5 ± 0.3
18:2n-6	53.7 ± 4.6	60.6 ± 3.8[b]	17.2 ± 5.2	27.5 ± 5.5[b]	12.6 ± 2.7	22.4 ± 5.1[c]	23.3 ± 2.4	27.2 ± 2.7[a]
18:3n-6	0.7 ± 0.3	0.9 ± 0.5	0.6 ± 0.3	0.7 ± 0.2			0.2 ± 0.1	0.2 ± 0.1
18:3n-3	0.8 ± 0.2	0.5 ± 0.2[a]	1.7 ± 0.8	1.3 ± 0.6	1.4 ± 0.4	1.5 ± 0.6	0.3 ± 0.1	0.2 ± 0.1
20:3n-6	0.6 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	0.5 ± 0.2[b]			2.6 ± 0.7	3.4 ± 0.7[a]
20:4n-6	5.1 ± 1.0	5.0 ± 1.2	1.3 ± 0.4	1.4 ± 0.5	0.6 ± 0.3	0.6 ± 0.3	7.4 ± 1.0	7.9 ± 2.1
20:5n-3	1.4 ± 0.6	0.4 ± 0.2[c]	0.5 ± 0.3	0.2 ± 0.1[b]			1.4 ± 0.6	0.5 ± 0.2[c]
22:4n-6							0.2 ± 0.0	0.3 ± 0.1[a]
22:5n-3			0.5 ± 0.2	0.2 ± 0.1[b]	0.5 ± 0.2	0.5 ± 0.2	0.9 ± 0.1	0.8 ± 0.2
22:6n-3	0.8 ± 0.2	0.4 ± 0.2[c]	1.6 ± 0.7	0.4 ± 0.3[b]	0.9 ± 0.4	0.4 ± 0.4[a]	4.9 ± 1.0	2.4 ± 0.9[c]
n-3	3.0 ± 0.8	1.3 ± 0.3[c]	4.3 ± 1.2	2.1 ± 0.5[c]	2.8 ± 0.3	2.4 ± 0.4[a]	7.5 ± 1.5	3.9 ± 0.7[c]
n-6	60.1 ± 4.5	67.2 ± 2.8[b]	19.4 ± 5.5	30.1 ± 5.9[b]	13.2 ± 2.8	23.0 ± 5.2[c]	33.7 ± 2.1	39.0 ± 1.6[c]
n-3/n-6	0.05 ± 0.01	0.02 ± 0.00[c]	0.23 ± 0.07	0.07 ± 0.02[c]	0.22 ± 0.05	0.11 ± 0.03[c]	0.23 ± 0.05	0.10 ± 0.02[c]

[a]$P < 0.05$. [b]$P < 0.01$. [c]$P < 0.001$.

tive deacylation–reacylation of PL probably maintains higher levels of n-3 fatty acids than would be expected on the basis of dietary data. However, the proportions of eicosapentaenoic and docosahexaenoic acids in erythrocyte and platelet lipids of vegans were much lower than in omnivores. They were also clearly lower than we have found in subjects consuming an otherwise usual mixed diet but not consuming any fish products (Ågren, J., unpublished data). This is probably caused by the higher intake of linoleic acid in vegans leading to a decreased incorporation and desaturation of α-linolenic acid. These results indicate that with very low intakes of long-chain n-3 fatty acids their amounts in membrane lipids are more easily altered by other dietary fatty acids, especially n-6 fatty acids, than with intakes obtained from usual mixed diet.

The mean intake of linolenic acid was higher in vegans than in omnivores. This difference was not seen in plasma lipids. This may be caused by the greater linoleic acid intake of vegans causing displacement of α-linolenic acid. It is also possible that the calculated total linolenic acid intake contains more γ-linolenic acid in vegans. The variation between subjects was also much greater in vegans. However, the amount of linolenic acid in the diet of vegans correlated with the proportion of α-linolenic acid in serum TG, CE, and PL, and also in erythrocyte phosphatidylethanolamine and PS (r = 0.71–0.77, $P < 0.05$ in all). This is in accordance with the results of Chan *et al.* (15), who also found increased levels of eicosapentaenoic and docosapentaenoic acids in individuals with increased α-linolenic acid intakes. Mantzioris *et al.* (16) have also shown that the proportions of long-chain n-3 fatty acids are increased in plasma lipids by a high α-linolenic acid intake. In the present study, the α-linolenic acid intake or its ratio to linoleic acid did not show any correlation with longer chain n-3 fatty acids. In fact, the lowest levels of long-chain n-3 fatty acids were found in those vegans having the greatest linolenic acid intakes and levels in serum and membrane lipids. If there really was a negative correlation, it would imply that dietary α-linolenic acid intake should accompany some factor preventing its elongation but not affecting linoleic acid metabolism. This does not seem very probable. It should be noted that quite high α-linolenic acid intakes are needed to increase the amounts of long-chain n-3 fatty acids, and the effect on docosahexaenoic acid seems to be very small (15,16). It is obvious that not much preformed long-chain n-3 fatty acids are needed to obscure the effect of α-linolenic acid. It is possible that the differences in the low intake of preformed long-chain n-3 fatty acids between vegans overshadow the effect of dietary α-linolenic acid. Small amounts of long-chain n-3 fatty acids could be derived from the seaweed products commonly included in the vegan diet. Whatever the reason, these results show the weak ability of α-linolenic acid to increase the amount of longer chain n-3 fatty acids when compared to direct intake of these fatty acids.

The proportion of nervonic acid in erythrocytes was significantly greater in vegans, the same trend also being observed in platelets. Nervonic acid is located almost exclusively in the sphingomyelin fraction of membrane lipids. The proportions of other sphingomyelin derived fatty acids, such

as behenic acid (20:0) and lignoceric acid (24:0), were similar in both groups. This indicates that there are differences in the fatty acid composition of sphingomyelin between vegans and omnivores. This might be some compensatory change, e.g., to maintain membrane physical properties.

There has been considerable controversy about the influence of a vegetarian diet on levels of arachidonic acid. The high linoleic acid intake may interfere with arachidonic acid metabolism by competition for esterification and by suppressing desaturases (17). The small amount of arachidonic acid present in a vegetarian diet may also decrease its amount in serum and membrane lipids (8). It could be speculated that a vegetarian diet could alleviate the symptoms of rheumatoid arthritis by depressing the eicosanoid production from arachidonic acid. The reported data of arachidonic acid level in vegetarians is, however, inconsistent, showing lower (6,8), higher (7), and similar (7,9) levels as in the omnivore controls in different lipid fractions. In the present study, the proportions of other long-chain n-6 fatty acids were generally greater in vegans, but arachidonic acid level differed only in erythrocyte PS, where it was greater in vegans. It is obvious that all vegetarian diets do not necessarily decrease the arachidonic acid content in serum and membrane lipids. It could be speculated that even the small increase in the amount of long-chain n-3 fatty acids in a vegetarian diet would decrease the amount of arachidonic acid in membranes. This could be of importance if a lowered level of arachidonic acid metabolism is desirable. The increase of 22-carbon n-6 fatty acids in vegans is probably a compensatory mechanism in response to the low levels of docosahexaenoic acid. Docosahexaenoic acid is necessary for normal development of the nervous system. However, the possible significance of low plasma and tissue levels of docosahexaenoic acid to changes in the health of adults is not known.

It could be concluded that similar kinds of differences as those found in the erythrocytes and platelet membranes are also present in the membranes of other tissues. This may cause alterations in membrane properties. It could be argued that vegan diet changes the eicosanoid formation due to lowered n-3 fatty acid levels. It is also possible that the characteristic membrane fatty acid composition of vegans could influence the activity of membrane enzymes and receptors as well as physical properties of the membrane itself. At present, little is known about the relationships between fatty acid composition and membrane function, and no direct conclusions can be made.

In addition to fatty acid intake, there are also many other differences in nutrient intakes between strict vegan and omnivore diets. Differences in the vitamin and trace element intakes might also have effects on lipid composition. However, the differences found in the fatty acid profiles between these groups were quite consistent with the dietary intakes of fatty acids. Thus, it is apparent that differences in the fatty acid intakes are the main cause for the observed differences in plasma and membrane lipids of vegans and omnivore controls.

REFERENCES

1. Farquhar, J.W., and Ahrens, Jr., E.H. (1963) *J. Clin. Invest. 42*, 675–685.
2. Hunter, D. (1990) in *Nutritional Epidemiology* (Willet, W., ed.), pp. 191–202, Oxford University Press, New York.
3. v. Houwelingen, A.C., Kester, A.D.M., Kromhout, D., and Hornstra, G. (1989) *Eur. J. Clin. Nutr. 43*, 11–20.
4. Sarkkinen, E.S., Ågren, J.J., Ahola, I., Ovaskainen, M.L., and Uusitupa, M.I.J. (1994) *Am. J. Clin. Nutr. 59*, 364–370.
5. Dwyer, J.T. (1991) *Annu. Rev. Nutr. 11*, 61–91.
6. Sanders, T.A.B., and Roshanai, F. (1992) *Eur. J. Clin. Nutr. 46*, 823–831.
7. Sanders, T.A.B., Ellis, F.R., Path, F.R.C., and Dickerson, J.W.T. (1978) *Am. J. Clin. Nutr. 31*, 805–813.
8. Phinney, S.D., Odin, R.S., Johnson, S.B., and Holman, R.T. (1990) *Am. J. Clin. Nutr. 51*, 385–392.
9. Melchert, H.U., Limsathayourat, N., Mihajlovic, H., Eichberg, J., Thefeld, W., and Rottka, H. (1987) *Atherosclerosis 65*,159–166.
10. Ling, W.H., and Hänninen, O. (1992) *J. Nutr. 122*, 924–930.
11. Ågren, J.J., Hänninen, O., Laitinen, M., Seppänen, K., Bernhardt, I., Fogelholm, L., Herranen, J., and Penttilä, I. (1988) *Lipids 23*, 924–929.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
13. Ågren, J.J., Julkunen, A., and Penttilä, I. (1992) *J. Lipid. Res. 33*, 1871–1876.
14. Glatz, J.F.C., Soffers, A.E.M.F., and Katan, M.B. (1989) *Am. J. Clin. Nutr. 49*, 269–276.
15. Chan, J.K., McDonald, B.E., Gerrard, J.M., Bruce, V.M., Weaver, B.J., and Holub, B.J. (1993) *Lipids 28*, 811–817.
16. Mantzioris E., James, M.J., Gibson, R.A., and Cleland, L.G. (1994) *Am. J. Clin. Nutr. 59*, 1304–1309.
17. Lands, W.E.M. (1986) *Fish and Human Health*, Academic Press, New York.

[Received May 12, 1994 and in revised form January 10, 1995; Revision accepted January 15, 1995]

CHANGE OF REFERENCES STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) "Temperature and Diet Effects on Trout," *Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) "Temperature and Diet Effects on Trout," in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., "Temperature and Diet Effects on Trout," *J. Biol. Chem.*, in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

AVAILABILITY OF FISH OIL TEST MATERIALS

The Fish Oil Test Materials Program is a cooperative effort of the National Institutes of Health (NIH) and the National Oceanic and Atmospheric Administration/Department of Commerce (NOAA/DOC). This program provides a long-term, consistent supply of quality-assured/quality-controlled test materials to researchers that facilitates the evaluation of ω-3 fatty acids in health and disease.

Current test materials available, in both capsules and bulk, include: (i) vacuum deodorized menhaden oil; (ii) ethyl esters of olive, corn, and safflower oils; (iii) commercial preparations of corn, olive, and safflower oil; (iv) n-3 ethyl ester concentrates; (v) EPA ethyl ester; and (vi) DHA ethyl ester.

To qualify to receive the fish oil test materials from the Program, the researcher must fill out an application. The applicant must: (i) have peer-reviewed research indicating the need for the requested materials, and (ii) submit a cor-

(continued on next page)

rectly completed application form and a signed waiver of liability. *The awarded materials are provided free of charge (researchers will pay shipping costs).*

Investigators may obtain further information and an application form from Fish Oil Test Materials Program, NIAAA, National Institutes of Health, 12501 Washington Ave., Flow Building, Room 2, Rockville, MD 20852 (Phone: Beth Aurecchia, 301-443-9643; Fax: 301-594-0035).

CALENDAR

June 1995

2nd International Congress of the International Society for the Study of Fatty Acids and Lipids, June 7–10, National Institutes of Health, Bethesda, Maryland, USA. Information: A.P. Simopolous, ISSFAL, 2001 S St., N.W., Suite 530, Washington, DC 20009 USA.

International Lecture Evening, SCI Oils and Fats Group, June 8, 1995, SCI Building, 14/15 Belgrave Sq., London, England. Prof. Karé Larsson to speak on "The Physical Properties of Lipids in Relation to Their Function in Foods." Information: Conference Secretariat, SCI, 14/15 Belgrave Sq., London SW1X 8PS, United Kingdom (Fax: 44-71-823-1698).

18th Nordic Lipid Symposium, June 18–21, Reykjavik, Iceland. Information: G. Lambertsen, Lipidforum Secretariat, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-325724).

August 1995

36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September 1995

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October 1995

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

American College of Nutrition 36th Annual Meeting, Oct. 13–15, 1995, Omni Shoreham Hotel, Washington, DC. Information: American College of Nutrition, c/o Hospital for Joint Disease, 301 E. 17th St., New York, NY 10003 (Fax: 212-777-1103).

Lipidforum Seminar: Lipids in Animal Nutrition, Oct. 25–26, 1995, Research Centre Foulum, Tjele (Viborg), Denmark. Information: G. Lambertsen, Lipidforum, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-32 57 24).

November 1995

XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

Leukotriene B_4 Stimulation of an Early Elevation of Phosphatidic Acid Mass in Human Neutrophils

Jen-sie Tou* and Thao Dola

Department of Biochemistry, Tulane Medical School, New Orleans, Louisiana 70112

ABSTRACT: The signal transduction pathway of leukotriene B_4 involves phospholipase D activation in cytochalasin B-primed neutrophils, but leukotriene B_4 stimulation of increased phosphatidic acid mass in neutrophils has not been demonstrated. Employing the NIH Image program, we have examined the effect of leukotriene B_4 on phosphatidic acid mass in human neutrophils incubated with or without cytochalasin B. Our results show that 0.15 μM leukotriene B_4 without cytochalasin B was capable of increasing phosphatidic acid mass in neutrophils by 2-fold after 5 s, 2.5-fold after 1 min, and 2-fold after 5 min incubation. Leukotriene B_3, leukotriene B_4, and leukotriene B_5 were equipotent stimuli for phosphatidic acid mass elevation. Leukotriene B_4 induced phosphatidylethanol formation at the expense of phosphatidic acid in cells preincubated with 0.25–1% ethanol, indicating phospholipase D activation. Cytochalasin B enhanced leukotriene B_4 stimulation of phosphatidic acid mass elevation and phosphatidylethanol formation. There were no measurable changes in 1,2-diglyceride mass after 5 s, but a 1.7-fold increase occurred after 1 min and declined thereafter. Leukotriene B_4 stimulation of [^{3}H]glycerol incorporation into phosphatidic acid, diglyceride and phosphatidylinositol was detectable after a 1-min incubation, suggesting increased *de novo* synthesis of these lipids. These results suggest that leukotriene B_4 stimulation of phospholipase D activity contributes to part of the early increased phosphatidic acid mass and that combined actions of stimulated phospholipases C and D, and *de novo* phosphatidic acid synthesis contribute to the total increased phosphatidic acid mass.
Lipids 30, 373–381 (1995).

Leukotriene B_4 (LTB_4) is a potent agonist for neutrophil chemotaxis (1). It also stimulates neutrophil metabolism. Its signal transduction pathway in neutrophils involves activation of phospholipases A_2 (2,3) and C (4), but LTB_4 stimulation of phospholipase D activity has been shown to require priming neutrophils by cytochalasin B (5). The products of phospholipid hydrolysis and their metabolites in turn serve as second messengers to amplify neutrophil metabolism. Activation of phospholipase A_2 leads to the synthesis of platelet-activating factor (PAF), which is an agonist for neutrophil chemotaxis and aggregation (6). Activation of phospholipase C results in the production of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and 1,2-diglyceride (DG). [Ins(1,4,5)P_3] raises cytosolic Ca^{+2} levels (7). Although DG is a protein kinase C activator, LTB_4 has not been shown to increase protein kinase C activity in neutrophils. This could be due to a rapid metabolism of DG to phosphatidic acid (PtdOH) and triglyceride (TG). LTB_4 has been shown to enhance ^{32}P-PtdOH formation in human neutrophils prelabeled with ^{32}Pi (8). It has also been demonstrated to induce [^{3}H]alkyl-PtdOH production in cytochalasin B primed rabbit peritoneal neutrophils, which were prelabeled with 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (5). However, increased PtdOH mass in neutrophils challenged with LTB_4 has not been demonstrated. In intact neutrophils, PtdOH can be formed from phosphorylation of DG by a DG kinase or from the hydrolysis of choline glycerophospholipid by a phospholipase D in the presence of cytochalasin B. In addition, it can also be derived from *de novo* synthesis. Indeed, LTB_4 has been shown to promote the incorporation of palmitic, but not arachidonic, acid into PtdOH (9).

In the present studies, we demonstrate that LTB_4 without the priming effect of cytochalasin B is capable of inducing a prompt (5 s) but transient (5 min) elevation of PtdOH mass in human neutrophils. Cytochalasin B enhanced PtdOH mass elevation induced by LTB_4. Our results suggest that LTB_4 stimulation of phospholipase D activity contributes to the prompt increase in PtdOH mass, and that combined actions of stimulated phospholipases C and D and *de novo* PtdOH synthesis contribute to the total increased PtdOH mass.

MATERIALS AND METHODS

Materials. Commercial sources of the reagents used are: PtdOH prepared from egg yolk lecithin, fatty acid-free bovine serum albumin (BSA), and LTB_4 from Sigma (St. Louis, MO); leukotriene B_3 (LTB_3), leukotriene B_5 (LTB_5), and 12-

*To whom correspondence should be addressed at the Department of Biochemistry SL 43, Tulane Medical Center, 1430 Tulane Ave., New Orleans, LA 70112.

Abbreviations: BSA, bovine serum albumin; CGPL, choline glycerophospholipid; DG, 1,2-diglyceride; EGPL, ethanolamine glycerophospholipid; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; LTB_3, leukotriene B_3 (5*S*,12*R*-dihydro-6-*cis*-8,10-*trans*-eicosatrienoic acid); LTB_4, leukotriene B_4, (5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid); 12-*epi*-LTB_4, 12-*epi*-leukotriene B_4; LTB_5, leukotriene B_5 (5*S*,12*R*-dihydro-6,14,17-*cis*-8,10-*trans*-eicosapentaenoic acid); PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PtdOH, phosphatidic acid; PtdEt, phosphatidylethanol; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; SF_1, solvent front of the first solvent; SF_2, solvent front of the second solvent; TG, triglyceride; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

epi-leukotriene B_4 (12-*epi*-LTB_4) from Cayman (Ann Arbor, MI); phosphatidylethanol (PtdEt) from Avanti Polar-Lipids (Alabaster, AL); Merck silica gel 60 plastic-backed plates from VWR (Philadelphia, PA); [1,2,3-^{3}H]glycerol (40 Ci/mmol) from Du Pont–New England Nuclear (Boston, MA); cytochalasin B from Aldrich (Milwaukee, WI); silica gel H glass plates from Analtech (Newark, DE). The sources of reagents for DG mass determinations were the same as previously described (10).

Preparation of human neutrophils. Neutrophils were isolated from heparinized peripheral blood from healthy volunteers as previously described (9). Cells were suspended in Dulbecco's balanced salt solution (11), pH 7.4, supplemented with 5 mM glucose at a concentration of 2×10^7 cells/mL. Cell counts were made in a hemocytometer, and cell viability was measured by Trypan blue exclusion. Cell preparations contained more than 95% neutrophils.

Incubation of neutrophils. LTB_4 or (other leukotrienes in ethanol) was evaporated at room temperature under vacuum by a rotary evaporator and suspended in Dulbecco's balance salt solution containing 4.0 mg of BSA per mL. Each incubation tube contained, in a final volume of 2 mL, 0.4 mg of BSA, 0–15 μM LTB_4, 0–1% ethanol ± 10 μg cytochalasin B (or equivalent amounts of dimethylsulfoxide), and 2×10^7 cells. Unless otherwise indicated, each experiment was started by a 10-min preincubation of cell suspensions at 37°C with 0–1% ethanol ± 10 μg cytochalasin B, and then incubations were started by adding LTB_4 or other leukotrienes to each tube. After 5 s–10 min at 37°C, incubations were stopped by adding 5 mL of methanol to each tube. Total lipids were extracted according to the original method of Bligh and Dyer (12). The chloroform layer was evaporated under vacuum by a rotary evaporator. The dried lipid residue was redissolved in chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene.

Thin-layer chromatography (TLC) of PtdOH. PtdOH was resolved from other lipids by a two-step one-dimensional TLC on plastic-backed silica gel 60 plates (20 × 20 cm). The plates were not treated with potassium oxalate. Each lipid extract from 2×10^7 cells was dissolved in 25 μL chloroform/methanol (2:1, vol/vol), and 18 μL (1.44×10^7 cell equivalents) was applied to the plate (20 × 10 cm) as a 1-cm streak. PtdOH standards from egg yolk lecithin in the range of 129–1290 pmoles were applied to the same plate as the lipid extracts. A total of 12 samples can be applied to one plate. One PtdOH standard (1290 pmoles) was applied as a 1-cm streak on a separate plate (5 × 10 cm). Both plates were first developed in ethyl acetate/acetic acid/isooctane (36:8:20, by vol) as described by Mallows and Bolton (13). After drying in a hood for one hour, the plate with PtdOH standard only was stained with iodine, and the PtdOH area was marked to serve as a guide for PtdOH mobility on the sample plate. In the first solvent system, PtdOH moved as a wide band with an R_f value of 0.61. The sample plate was cut at 0.5-cm above the PtdOH standard. It was then turned 180° and developed in the second solvent system consisting of chloroform/methanol/28% ammonia (50:20:4, by vol) until the solvent moved to the sample origin of the plate. PtdOH appeared as a narrow band and was well separated from other lipids.

For PtdEt determination from cells without cytochalasin B, lipid extracts from duplicate incubations were combined, and each lipid sample from 2.88×10^7 cell equivalents was applied to the TLC plate as a 1-cm streak. PtdEt standards in the range of 129–1290 pmoles were applied to the same plate. PtdEt was separated from other lipids in chloroform/methanol/acetic acid (45:5:5, by vol) as described by Olson *et al.* (14).

Quantitation of PtdOH and PtdEt. After drying in a hood for 30 min, the chromatogram was stained with iodine vapor for 5 min and then sprayed with 10% cupric sulfate in 8% phosphoric acid (15). It was then heated in a 100°C oven for 20 min and for 10 additional min at 120°C. The bands of PtdOH and PtdEt were scanned on a DeskScan II and analyzed on a Macintosh LCII computer using the public domain NIH Image 1.56b18 program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, Springfield, VA; part number PB93-504868). The mass amounts of PtdOH and PtdEt were expressed as pmoles ± SEM per 10^7 cells from three separate experiments.

Recovery of PtdOH extracted in neutral solvents by the method of Bligh and Dyer (12). PtdOH standards in the range of 129–1290 pmoles in 5 mL chloroform were added to tubes containing 5 mL methanol, 2.5 mL chloroform, and 2 mL incubation buffer or 2 mL incubation buffer containing 2×10^7 neutrophils. After 20 min at room temperature, the mixture was partitioned with 2.5 mL each of chloroform and water. The chloroform layer was evaporated under vacuum by a rotary evaporator. The dried lipid residue was redissolved in chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene. PtdOH was resolved by the two-step one-dimensional TLC, scanned and quantitated as described above.

DG mass determination. DG levels in total lipid extracts from cells incubated with or without 0.15 mM LTB_4 were determined by using *Escherichia coli* DG kinase to convert DG into PtdOH (16). DG mass was expressed as pmoles ± SEM per 10^7 cells from three separate experiments.

Incorporation of [^{3}H]glycerol into lipids. Each incubation tube contained, in a volume of 2 mL, 0.4 mg BSA, 10 mCi (1.1 mM) of [1,2,3-^{3}H]glycerol, ±0.15 μM LTB_4, and 2×10^7 cells. Incubation tubes and cells were kept separately at 37°C for 5 min, and then incubations were started by adding 1 mL of cells (2×10^7) to each tube. After 1–10 min at 37°C, incubations were stopped by adding 5 mL of methanol to each tube containing 2 mL incubation mixture. Total lipids were extracted according to the method of Bligh and Dyer (12). The lipid residue from 2×10^7 cells in each tube was dissolved in 50 μL chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene, and each 15 μL representing 6×10^6 cell equivalents was used for resolution of

phospholipids or neutral glycerolipids. Phospholipids were separated by two-dimensional TLC, and neutral glycerolipids were separated by one-dimensional TLC on silica gel H glass plates (17). The radioactivity of each lipid was counted by liquid scintillation spectrometry and expressed as dpm ± SEM per 6×10^6 cells from three experiments.

RESULTS

TLC of neutrophil lipid extracts and PtdOH standards. PtdOH in lipid extracts was separated from other lipids by a two-step one-dimensional TLC system. Figure 1A demonstrates two chromatograms scanned with DeskScan II. The left chromatogram shows that in the first solvent PtdOH standard and PtdOH in a lipid extract from 2×10^7 cells moved as a wide band with an R_f value of 0.61. However, after developing in the second solvent both PtdOH standard and PtdOH in the lipid extract appeared as a narrow band (right chromatogram). For PtdOH mass measurement, a standard curve was constructed on the same plate as the lipid extracts. The intensity of PtdOH was greatly enhanced when only the PtdOH areas were scanned. Figure 1B is a representative chromatogram demonstrating the effect of LTB_4 on PtdOH mass in neutrophils preincubated with cytochalasin B and different concentrations of ethanol.

PtdOH can be quantitatively recovered from neutrophils under the experimental conditions. The recovery of PtdOH extracted in neutral solvents by the method of Bligh and Dyer (12) was examined. The results shown in Figure 2 indicate that the recovery for PtdOH standards varied with the amounts of PtdOH in each tube. An average of PtdOH recovery was 50% for 129 pmoles, and it reached 90% for 1290 pmoles. Also shown in Figure 2 is that a quantitative recovery of PtdOH was achieved at all concentrations tested if PtdOH standards were extracted together with 2×10^7 cells. The results indicate that PtdOH was quantitatively recovered from neutrophils under the experimental conditions.

We found that staining the chromatogram in iodine vapor enhanced the intensity of lipids charred with $CuSO_4/H_3PO_4$ by twofold (data not shown), thereby enhancing the sensitivity of lipid analysis by using the NIH Image program. This method measured PtdOH from egg yolk lecithin with a linear range of 129–1290 pmoles. Although the values of PtdOH determined by the present method was 25–30% lower than those

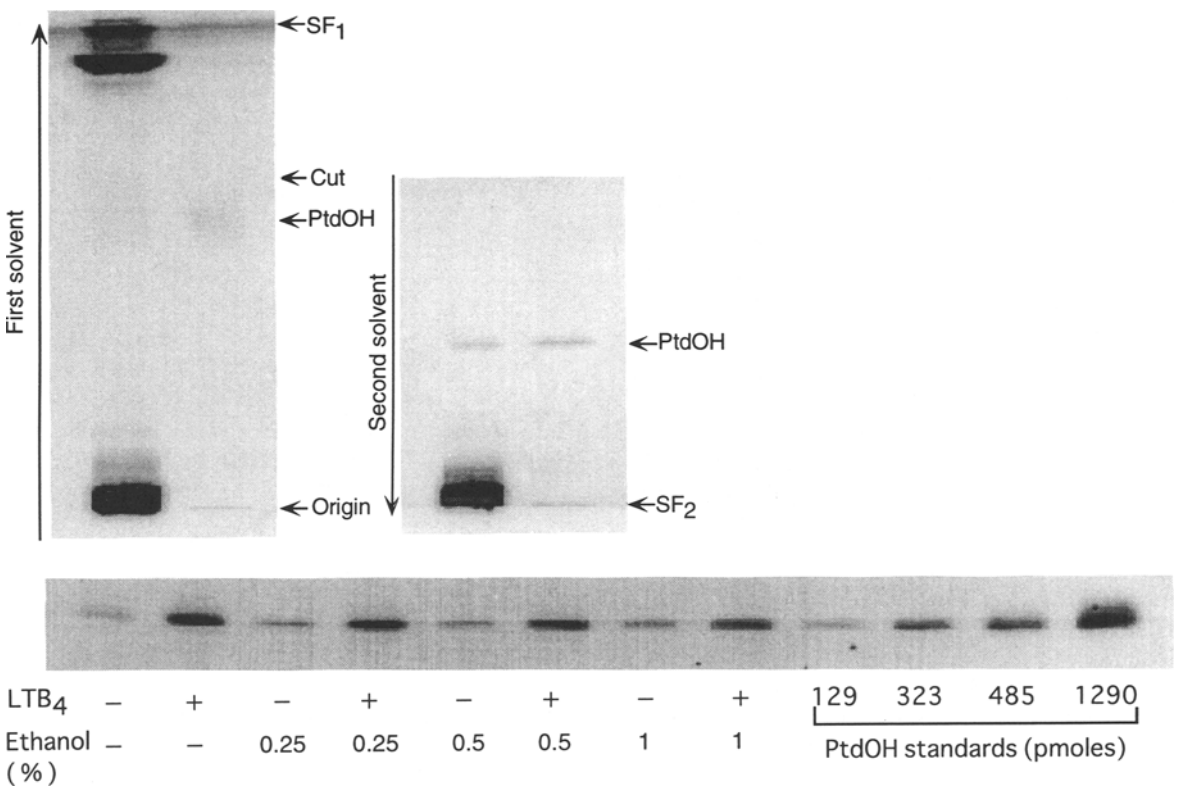


FIG. 1. Thin-layer chromatography (TLC) of neutrophil lipid extracts and phosphatidic acid (PtdOH) standards. A. PtdOH in neutrophils was separated from other lipids by a two-step one-dimensional TLC. Neutrophil lipid extract from 2×10^7 cells and a PtdOH standard (1290 pmoles) were each applied to two TLC plates. One of the plates (left) was only developed in the first solvent. The chromatograms were charred and scanned as described in the Materials and Methods section. B. Lipid extracts (each from 1.44×10^7 cells) were from cytochalasin B-primed neutrophils incubated with 0.15 μM leukotriene B_4 (LTB_4) for 1 min in the presence of 0–1% ethanol. PtdOH in neutrophil extracts and PtdOH standards were applied to the same plate. After the two-step one-dimensional TLC, the plate was charred, and only the PtdOH areas were scanned with DeskScanII as described in the Materials and Methods section.

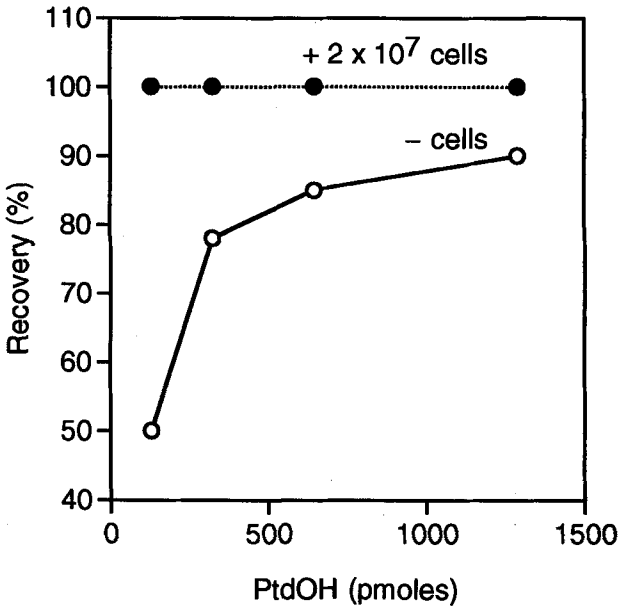


FIG. 2. Recovery of PtdOH extracted in neutral solvents by the method of Bligh and Dyer (Ref. 12). PtdOH standards in the range of 129–1290 pmoles in 5 mL chloroform were added to tubes containing 5 mL methanol, 2.5 mL chloroform, and 2 mL incubation buffer or 2 mL incubation buffer containing 2×10^7 neutrophils. Lipids were extracted in neutral solvents by the method of Bligh and Dyer (Ref. 12), and PtdOH was separated and quantitated as described in the Materials and Methods section. Data expressed are average values from two experiments. Abbreviations as in Figure 1.

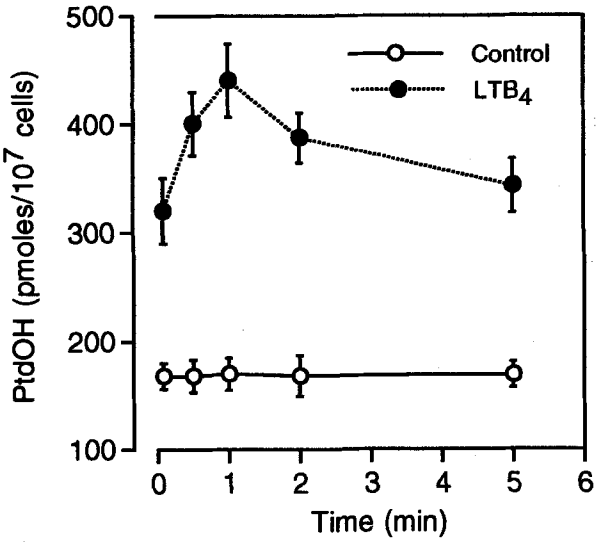


FIG. 3. Time course of LTB_4 effect on PtdOH mass. Each incubation tube contained, in a final volume of 2 mL, 0.4 mg of bovine serum albumin (BSA) ± 0.15 μM LTB_4, and 2×10^7 neutrophils. Neutrophils were preincubated for 10 min at 37°C, then incubations were started by adding 0.15 μM LTB_4 or BSA to each tube. After 5 to 10 min at 37°C, incubations were stopped by adding 5 mL methanol to each tube. Lipids were extracted, and PtdOH was separated and quantitated as described in the Materials and Methods section. Data are expressed as pmoles ± SEM per 10^7 cells from three separate experiments. Abbreviations as in Figure 1.

determined by phosphate analysis (10), the extent of LTB_4 stimulation of PtdOH mass increase was similar. The lower values obtained by the present method may reflect a lower degree of unsaturation of neutrophil PtdOH than that of the PtdOH standards, as unsaturated lipids are more sensitive to both iodine staining and to $CuSO_4/H_3PO_4$ charring than saturated lipids.

LTB_4 elicits a prompt rise in PtdOH mass. Figure 3 depicts the time course of PtdOH mass increase in neutrophils challenged with 0.15 μM LTB_4 without cytochalasin B. The PtdOH mass (pmoles per 10^7 cells) increased from 168 ± 12 to 320 ± 30 after 5 s, reaching a maximum of 440 ± 34 after 1 min. It declined to 343 ± 25 after 5 min and returned to basal levels after a 10-min incubation (data not shown).

*LTB_3 and LTB_5, but not 12-*epi*-LTB_4 increase PtdOH mass.* The specificity of LTB_4 stimulation of PtdOH mass increase was examined by incubating neutrophils with 0.24 μM LTB_3, LTB_4, LTB_5, or 12-*epi*-LTB_4 for 1 min. PtdOH content was measured after separating PtdOH from other lipids by the two-step one-dimensional TLC. The results summarized in Table 1 show that LTB_3, LTB_4, and LTB_5 were equally active in stimulation of PtdOH mass increase under the experimental conditions. However, the structural analog 12-*epi*-LTB_4 was ineffective on PtdOH mass elevation.

LTB_4 stimulation of PtdOH mass elevation is concentration-dependent. Figure 4 shows LTB_4 stimulation of PtdOH mass elevation in a concentration-dependent manner. The basal levels of PtdOH mass were 182 ± 12 pmoles per 10^7 cells. Incubation of neutrophils with 0.015 μM LTB_4 for 1 min increased PtdOH mass by 1.7-fold. PtdOH mass increase reached a maximum of 2.5-fold at 0.15 μM LTB_4. The effects of LTB_4 concentrations lower than 0.015 μM were not examined.

Activation of phospholipase D contributes to part of the early PtdOH mass elevation. The prompt rise in PtdOH mass induced by LTB_4 suggests phospholipase D activation. In neutrophils, phospholipase D catalyzes the hydrolysis of choline glycerophospholipid, generating PtdOH and choline

TABLE 1
Effect of LTB_3 and LTB_5 on Phosphatidic Acid (PtdOH) Mass[a]

Leukotrienes (0.24 μM)	PtdOH (pmoles/10^7cells)
None	178 ± 15
LTB_4	435 ± 36
12-*epi*-LTB_4	195 ± 16
LTB_3	471 ± 42
LTB_5	444 ± 29

[a]Each incubation tube contained, in a final volume of 2 mL, 0.4 mg bovine serum albumin ± 0.24 μM leukotriene, and 2×10^7 neutrophils. Cells were preincubated for 10 min at 37°C; then incubations were started by adding leukotriene B_3 (LTB_3), leukotriene B_4 (LTB_4), or leukotriene B_5 (LTB_5) to each tube. After 1 min at 37°C, PtdOH was extracted, separated, and quantitated as described in the Materials and Methods section. Data are expressed as pmoles ± SEM per 10^7 cells from three separate experiments.

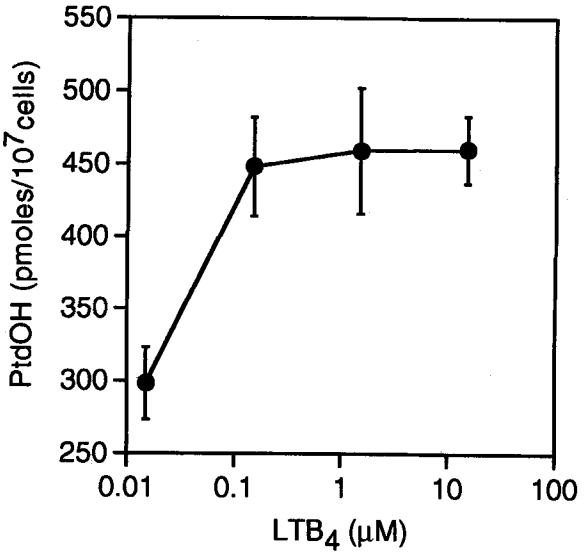


FIG. 4. Effect of LTB_4 concentration on PtdOH mass elevation. The incubation conditions and the abbreviations are the same as those in Figure 1, except that incubations were started by adding 0.015–15 μM LTB_4 or bovine serum albumin. After 1 min at 37°C, incubations were stopped by adding 5 mL methanol to each tube. Lipids were extracted, and PtdOH was separated and quantitated as described in the Materials and Methods section. Data are expressed as pmoles ± SEM per 10^7 cells from three separate experiments. The basal levels of PtdOH mass in neutrophils were 182 ± 12 pmoles per 10^7 cells.

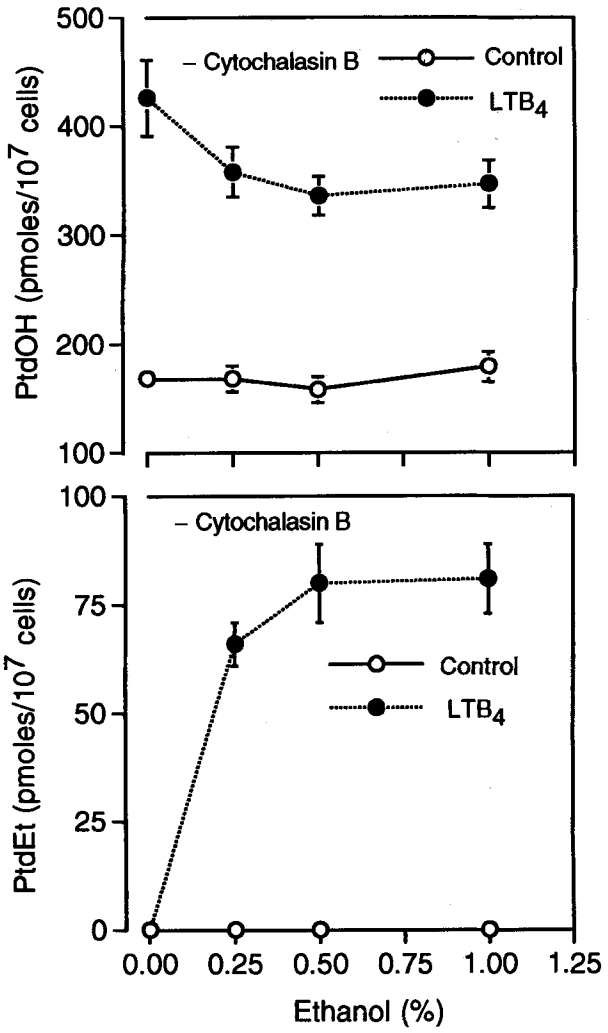


FIG. 5. Effect of LTB_4 on PtdOH and phosphatidylethanolamine (PtdEt) production in neutrophils without cytochalasin B. The incubation conditions (and abbreviations) were the same as those in Figure 1, except that cells were preincubated with 0–1% ethanol for 10 min at 37°C. Each tube was then further incubated with 0.15 μM LTB_4 for 1 min. PtdOH and PtdEt were extracted, separated, and quantitated as described in the Materials and Methods section. Data are expressed as pmoles ± SEM per 10^7 cells from three separate experiments.

(18,19). This enzyme also catalyzes the transfer of the phosphatidyl group to ethanol, propanol, or butanol, forming PtdEt or other phosphatidylalcohols (20). PtdEt is more metabolically stable than PtdOH and has been used for assay of phospholipase D activity in intact cells. It required the priming effect of cytochalasin B for the stimulation of phospholipase D by chemotactic peptide in human neutrophils (17) and by LTB_4 in rabbit peritoneal neutrophils (5). In the present study, the effect of LTB_4 on the changes in the mass amounts of PtdOH and PtdEt in cells with or without cytochalasin B was measured.

Figure 5 shows that 0.5–1% ethanol induced a maximum reduction in PtdOH mass elevation by 19% in cells incubated with 0.15 μM LTB_4 without cytochalasin B. It also demonstrates that decreased PtdOH mass paralleled with increased PtdEt formation. Because 1% ethanol had no effect on phospholipase C activity in human neutrophils (21), these results suggest that LTB_4 activation of phospholipase D contributes to part of the early increase in PtdOH mass induced by LTB_4 without cytochalasin B.

As demonstrated in Figure 6, LTB_4 induced a greater increase in PtdOH mass in cells primed with cytochalasin B than in primed cells without cytochalasin B (Fig. 5). In cytochalasin B-primed cells, ethanol (0.25–1%) inhibition of PtdOH mass elevation and stimulation of PtdEt formation were more pronounced than in cells without cytochalasin B, indicating that cytochalasin B enhanced the expression of phospholipase D activity in neutrophils (5,18,22).

DG mass elevation contributes to part of the increased PtdOH mass. LTB_4 has been shown to induce Ins(1,4,5) P_3 formation, indicating phospholipase C activation (4). Part of the DG derived from phospholipase C action is expected to be phosphorylated to form PtdOH by DG kinase. If the prompt PtdOH mass elevation is partly derived from increased DG mass, an increased DG mass is expected to occur after a 5 s challenge of cells with 0.15 μM LTB_4. However, Figure 7 shows that there was no measurable increase in DG mass after 5 s, a time in which a twofold increase in PtdOH was demonstrated (Fig. 1). DG mass was increased by 1.7-fold after 1 min; then it declined to basal levels (93 ± 7 pmoles per 10^7 cells) after 10 min. The results shown in Figure 7 suggest that increased DG mass may contribute to part

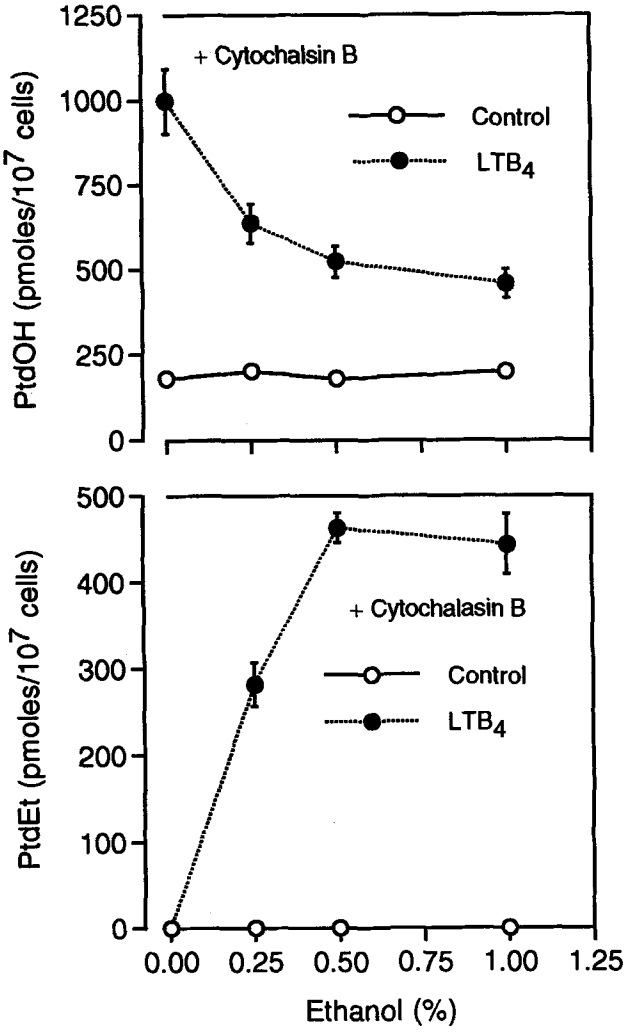


FIG. 6. Effect of LTB_4 on PtdOH and PtdEt production in neutrophils preincubated with cytochalasin B. The incubation conditions were the same as those in Figure 1, except that cells were preincubated for 10 min at 37°C with 0–1% ethanol and 10 μg cytochalasin B or equivalent amount of dimethylsulfoxide. The tubes were then further incubated with 0.15 μM LTB_4 for 1 min. PtdOH and PtdEt were separated and quantitated as described in the Materials and Methods section. Data are expressed as pmoles ± SEM per 10^7 cells from three separate experiments. Abbreviations as in Figures 1 and 5.

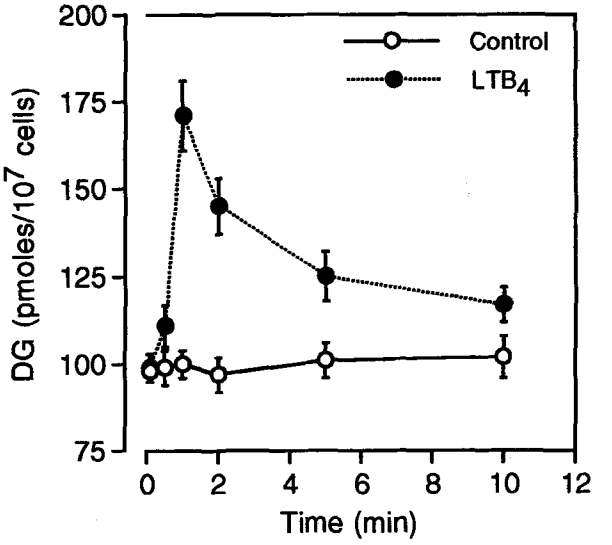


FIG. 7. Time course of LTB_4 effect on 1,2-diglyceride (DG) mass. The incubation conditions (and abbreviations) were the same as those in Figure 1. Total DG mass was measured by using *Escherichia coli* DG kinase as described in the Materials and Methods section. DG mass is expressed as pmoles ± SEM per 10^7 cells from three separate experiments.

of the PtdOH accumulation induced by LTB_4 after 1-min incubation.

LTB_4 stimulates de novo *PtdOH synthesis.* The effect of LTB_4 on *de novo* PtdOH synthesis was examined by measuring [^{3}H]glycerol incorporation into glycerolipids. Due to the low incorporation rate of exogenous [^{3}H]glycerol into PtdOH during 5–30 s of incubation, glycerol incorporation into phospholipids was measured after 1–10 min incubation. Figure 8 demonstrates LTB_4 stimulation of glycerol incorporation into PtdOH, DG, and phosphatidylinositol (PtdIns), suggesting increased *de novo* synthesis of these glycerolipids. LTB_4 induced a twofold increase in glycerol incorporation into PtdOH, DG, and PtdIns during 1–2-min incubations. The increased glycerol incorporation into PtdOH and DG started to decline after longer periods of incubation, but a twofold increase in PtdIns radioactivity remained even after a 10-min incubation. Phosphate analysis (10) could not detect the changes in PtdIns mass during the neutrophil–LTB_4 interaction. The changes in the radioactivity of choline glycerophospholipid (CGPL), ethanolamine glycerophospholipid (EGPL), and TG were insignificant.

DISCUSSION

The present study presents a convenient method for the separation of PtdOH from neutrophil lipid extract. Bocckino *et al.* (23) developed a two-step one-dimensional TLC for separation of PtdOH from hepatocyte extracts. However, it had a longer developing time than the present method for PtdOH separation. In the present method, prestaining the developed chromatogram with iodine enhanced the intensity of PtdOH charred with $CuSO_4/H_3PO_4$ twofold. PtdOH mass can be quantitatively measured on the NIH Image program. Under the experimental conditions, a 100% recovery of PtdOH from neutrophils could be achieved. By using this method, the present study has demonstrated that LTB_4 is capable of inducing a prompt (5 s) but transient (5 min) PtdOH mass accumulation in human neutrophils without the priming effect of cytochalasin B. Phospholipase D activation appears to contribute to part of the early increased PtdOH mass, as evidenced by PtdEt formation at the expense of PtdOH. Cytochalasin B enhanced PtdOH mass elevation induced by LTB_4. As cytochalasin B had no effect on phospholipase C activity (4,22), the results obtained by measuring the mass amounts of PtdOH and PtdEt support the finding that cytocha-

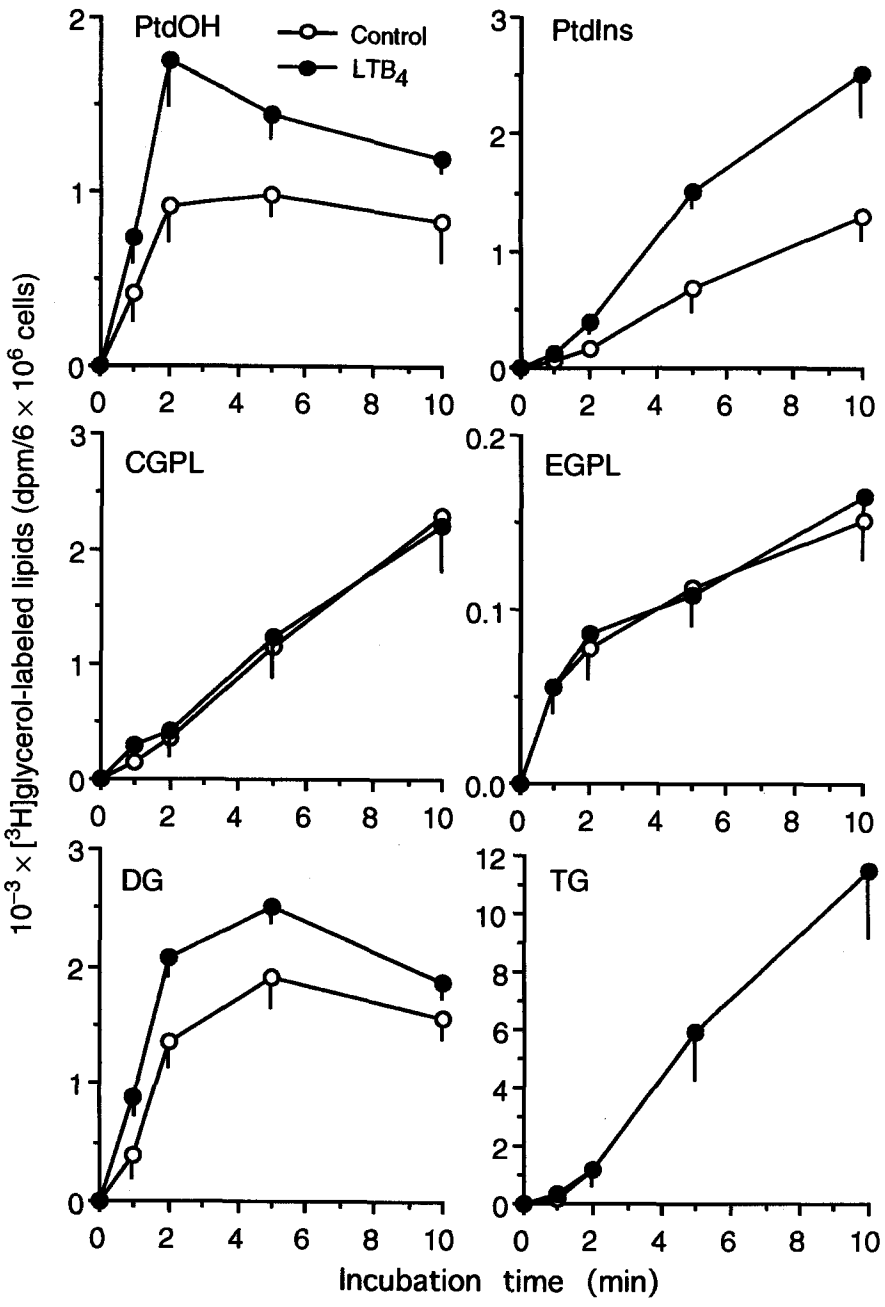


FIG. 8. Time course of LTB_4 effect on [^{3}H]glycerol incorporation into glycerolipids. Incubation conditions, lipid extraction and separation, and radioactivity measurements were described in the Materials and Methods section. The radioactivity of each lipid was expressed as dpm ± SEM per 6×10^6 cells from three experiments. Abbreviations as in Figures 1 and 7. PtdIns, phosphatidylinositol; CGPL, choline glycerophospholipid; EGPL, ethanolamine phospholipid; TG, triglyceride.

lasin B enhanced the expression of phospholipase D activity in human neutrophils (18,22) and in rabbit peritoneal neutrophils (5). The mechanism of cytochalasin B potentiation of phospholipase D activity in agonist-activated neutrophils is not well understood. Phospholipase D in neutrophils requires Ca^{2+} and small molecular weight GTP-binding protein for activity (24,25). It has been hypothesized that cytochalasin B may facilitate the availability of Ca^{2+} to phospholipase D (22).

Because LTB_4 has been shown to stimulate Ins(1,4,5) P_3 generation without cytochalasin B (4), part of the increased PtdOH mass may be derived from phospholipase C activation producing DG, which is subsequently phosphorylated to form

PtdOH. Although the changes in DG mass after 5 s were undetectable during the neutrophil–LTB_4 interaction, it is possible that the enzymatic method for DG mass determination is unable to detect the early small changes in DG mass.

The effect of LTB_4 is stereospecific because the structural analog 12-*epi*-LTB_4 had no effect on PtdOH levels. However, LTB_3, LTB_4, and LTB_5 were equipotent stimuli for PtdOH mass elevation. The effect of these leukotrienes on the changes in PtdOH mass appears to be at variance with their effects on chemotaxis and degranulation. A recent study (26) showed that LTB_3 was as active as LTB_4 in inducing neutrophil chemotaxis and lysozyme release from cytochalasin B-treated neutrophils. However, LTB_5 was shown to be a weaker stimulus of chemotaxis (26,27) and of lysozyme release (26) than LTB_4.

LTB_4 stimulation of glycerol incorporation into PtdOH, DG, and PtdIns suggests increased *de novo* synthesis of these lipids. Our earlier study (9), showing LTB_4 stimulation of palmitic, but not arachidonic, acid incorporation into PtdOH also suggests increased PtdOH synthesis. Enhanced PtdIns synthesis by LTB_4 may serve to replenish this molecule after its conversion to phosphatidylinositol 4,5-bisphosphate, which undergoes increased hydrolysis by phospholipase C (4) and increased transformation into phosphatidylinositol 3,4,5-triphosphate (28).

The function of PtdOH accumulation during the neutrophil–LTB_4 interaction deserves further study. Studies using cytochalasin B-treated neutrophils have demonstrated the association of degranulation (5,19) and superoxide anion production (29,30) with the formation of phospholipase D-derived PtdOH, which is a mixture of diacyl and alkylacyl species. However, PtdOH alone does not seem to be sufficient or necessary for initiating superoxide generation (31,32). The synergy of diacyl-PtdOH and diacylglycerol appears to be important in a cell-free system for NADPH oxidase activation (33). A recent study using neutrophils adherent to fibrinogen has shown that diradylglycerol (a mixture of diacylglycerol and alkylacylglycerol) derived from phospholipase D activation plays an important role in degranulation and oxidant release (34). In cell-free systems of other tissues, diacyl-PtdOH has been shown to induce protein phosphorylation (35) and activation of phosphatidylinositol 4-phosphate [PtdIns(4)P] 5-kinase (36,37). LTB_4 has been shown to promote phosphorylation of a 64-kD protein (38) and activation of a PtdIns(4)P 5-kinase (39) in neutrophils. It remains to be established whether the prompt rise in PtdOH mass induced by LTB_4 in these cells plays a role in these events.

ACKNOWLEDGMENT

This work was supported by a Research Grant from the American Heart Association–Louisiana Affiliate, Inc.

REFERENCES

1. Klickstein, L.B., Shapleigh, C., and Goetzl, E.J. (1980) "Lipoxygenation of Arachidonic Acid as a Source of Polymorphonuclear Leukocyte Chemotactic Factors in Synovial Fluid and Tissue in Rhumatoid Arthritis and Spondyloarthritis," *J. Clin. Invest. 66,* 1166–1170.
2. Tessner, T.G., O'Flaherty, J.T., and Wykle, R.L. (1989) "Stimulation of Platelet-Activating Factor Synthesis by a Nonmetabolizable Bioactive Analog of Platelet-activating Factor and Influence of Arachidonic Acid Metabolites," *J. Biol. Chem. 264,* 4794–4799.
3. Billah, M.M., Bryant, R.W., and Siegel, M. (1985) "Lipoxygenase Products of Arachidonic Acid Modulate Biosynthesis of Platelet-Activating Factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine)," *J. Biol. Chem. 260,* 6899–6906.
4. Verghese, M.W., Charles, L., Jakoi, L., Dillon, S.B., and Snyderman, R. (1987) "Role of a Guanine Nucleotide Regulatory Protein in the Activation of Phospholipase C by Different Chemoattractants," *J. Immunol. 138,* 4374–4380.
5. Kanaho, Y., Kanoh, H., Saitoh, K., and Nozawa, Y. (1991) "Phospholipase D Activation by Platelet-Activating Factor, Leukotriene B_4, and Formyl-Methionyl-Leucyl-Phenylalanine in Rabbit Neutrophils," *J. Immunol. 146,* 3536–3541.
6. Shaw, J.O., Pinckard, R., Ferrigni, K.S., McManus, L.M., and Hanahan, D.J. (1981) "Activation of Human Neutrophils with 1-*O*-Hexadecyl/Octadecyl-2-Acetyl-*sn*-Glyceryl-3-Phosphocholine (platelet-activating factor)," *J. Immunol. 127,* 1250–1255.
7. Berridge, M.J. (1987) "Inositol Trisphosphate and Diacylglycerol: Two Interacting Second Messengers," *Ann. Rev. Biochem. 56,* 159–193.
8. Omann, G.M., Traynor, A.E., Harris, A.L., and Sklar, L.A. (1987) "LTB_4-Induced Activation Signals and Responses in Neutrophils Are Short-Lived Compared to Formylpeptide," *J. Immunol. 138,* 2626–2632.
9. Tou, J.-S., and Healy, S. (1991) "Leukotriene B_4 Promotes Phospholipid Acylation in Human Neutrophils," *Lipids 26,* 327–330.
10. Tou, J.-S., Jeter, J.R., Jr., Dola, C.P., and Venkatesh, S. (1991) "Accumulation of Phosphatidic Acid Mass and Increased *de novo* Synthesis of Glycerolipid in Platelet-Activating Factor-Activated Human Neutrophils," *Biochem. J. 280,* 625–629.
11. Dawson, R.M.C., Elliott, D.C., Elliott, W.H., and Jones, K.M. (1986) "pH, Buffers, and Physiological Media," in *Data for Biochemical Research,* 3rd edn., p. 447, Clarendon Press, Oxford.
12. Bligh, E.G., and Dyer, W.J. (1959) "A Rapid Method of Total Lipid Extraction and Purification," *Can. J. Biochem. Physiol. 37,* 911–917.
13. Mallows, R.S.E., and Bolton, T.B. (1987) "Relationship Between Stimulated Phosphatidic Acid Production and Inositol Lipid Hydrolysis in Intestinal Longitudinal Smooth Muscle," *Biochem. J. 244,* 763–768.
14. Olson, S.C., Tyagi, S.R., and Lambeth, J.D. (1990) "Fluoride Activates Diradylglycerol and Superoxide Generation in Human Neutrophils via PLD/PA Phosphohydrolase-Dependent and -Independent Pathways," *FEBS Lett. 272,* 19–24.
15. Baron, C.B., and Coburn, R.F. (1984) "Comparison of Two Copper Reagents for Detection of Saturated and Unsaturated Neutral Lipids by Charring Densitometry," *J. Liq. Chromatogr. 7,* 2793–2801.
16. Preiss, J.E., Loomis, C.R., Bell, R.M., and Niedel, J.E. (1987) "Quantitative Measurement of *sn*-1,2-Diacylglycerols," *Methods Enzymol. 141,* 294–300.
17. Tou, J.-S. (1987) "Platelet-Activating Factor Modulates Phospholipid Acylation in Human Neutrophils," *Lipids 22,* 333–337.

18. Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W., and Siegel, M.I. (1989) "Phosphatidylcholine Hydrolysis by Phospholipase D Determines Phosphatidate and Diglyceride Levels in Chemotactic Peptide-Stimulated Human Neutrophils," *Biol. Chem. 264,* 17069–17077.
19. Cockcroft, S. (1992) "G-Protein-Regulated Phospholipases C, D and A_2-Mediated Signalling in Neutrophils," *Biochem. Biophys. Acta 1113,* 135–160.
20. Dawson, R.M.C. (1967) "The Formation of Phosphatidylglycerol and Other Phospholipids by the Transferase Activity of Phospholipase D," *Biochem. J. 102,* 205–210.
21. Nilsson, E., Andersson, T., Fällman, M., Rosendahl, K., and Palmbold, J. (1992) "Effects of Ethanol on the Chemotactic Peptide-Induced Second Messenger Generation and Superoxide Production in Polymorphonuclear Leukocytes," *J. Inf. Dis. 166,* 854–860.
22. Mullmann, T.J., Cheewatrakoolpong, B., Anthes, J.C., Siegel, M.I., Egan, R.W., and Billah, M.M. (1993) "Phospholipase C and Phospholipase D are Activated Independently of Each Other in Chemotactic Peptide-Stimulated Human Neutrophils," *J. Leuk. Biol. 53,* 630–635.
23. Bocckino, S.B., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987) "Phosphatidate Accumulation in Hormone-Treated Hepatocytes via a Phospholipase D Mechanism," *J. Biol. Chem. 262,* 15309–15315.
24. Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C., and Sternweis, P.C. (1993) "ADP-Ribosylation Factor, a Small GTP-Dependent Regulatory Protein, Stimulates Phospholipase D Activity," *Cell 75,* 1137–1144.
25. Bowman, E.P., Uhlinger, D.J., and Lambeth, J.D. (1993) "Neutrophil Phospholipase D is Activated by a Membrane-Associated Rho Family Small Molecular Weight GTP-Binding Protein," *J. Biol. Chem. 268,* 21509–21512.
26. Soyombo, O., Spur, B.W., Soh, C., and Lee, T.H. (1993) "Structure/Activity Relationship of Leukotriene B_4 and Its Structural Analogues in Chemotactic, Lysosomal-Enzyme Release and Receptor-Binding Assays," *Eur. J. Biochem. 218,* 59–66.
27. Goldman, D.W., Pickett, W.C., and Goetzl, E.J. (1983) "Human Neutrophil Chemotactic and Degranulating Activities of Leukotriene B_5 (LTB_5) Derived from Eicosapentaenoic Acid," *Biochem. Biophys. Res. Commun. 117,* 282–288.
28. Traynor-Kaplan, A.E., Thompson, B.L., Harris, A.L., Taylor, P., Omann, G. M., and Sklar, L.A. (1989) "Transient Increase in Phosphatidylinositol 3,4-Bisphosphate and Phosphatidylinositol Trisphosphate During Activation of Human Neutrophils," *J. Biol. Chem. 264,* 15668–15673.
29. Rossi, F., Grzeskowiak, M., Della Bianca, V., Calzetti, F., and Gandini, G. (1990) "Phosphatidic Acid and Not Diacylglycerol Generated by Phospholipase D Is Functionally Linked to the Activation of the NADPH Oxidase by FMLP in Human Neutrophils," *Biochem. Biophys. Res. Commun. 168,* 320–327.
30. Thompson, N.T., Tateson, J.E., Randall, R.W., Spacey, G.D., Bonser, R.W., and Garland, L.G. (1990) "The Temporal Relationship Between Phospholipase Activation, Diradylglycerol Formation and Superoxide Production in the Human Neutrophil," *Biochem. J. 271,* 209–213.
31. Kessels, G.C., Roos, D., and Verhoeven, A.J. (1991) "fMet-Leu-Phe-Induced Activation of Phospholipase D in Human Neutrophils," *J. Biol. Chem. 266,* 23152–23156.
32. Bauldry, S.A., Elsey, K.L., and Bass, D.A. (1992) "Activation of NADPH Oxidase and Phospholipase D in Permeabilized Human Neutrophils," *J. Biol. Chem. 267,* 25141–25152.
33. Quallione-Mann, D., Agwu, D.E., Ellsnberg, M.D., McCall, C.E., and McPhail, L.C. (1993) "Phosphatidic Acid and Diacylglycerol Synergize in a Cell-Free System for Activation of NADPH Oxidase from Human Neutrophils," *J. Biol. Chem. 268,* 23843–23849.
34. Suchard, S.J., Nakamura, T., Abe, A., Shayman, J.A., and Boxer, L.A. (1994) "Phospholipase D-Mediated Diradylglycerol Formation Coincides With H_2O_2 and Lactoferrin Release in Adherent Human Neutrophils," *J. Biol. Chem. 269,* 8063–8068.
35. Bocckino, S.B., Wilson, P.B., and Exton, J.H. (1991) "Phosphatidate-Dependent Protein Phosphorylation," *Proc. Natl. Acad. Sci. USA 88,* 6210–6213.
36. Moritz, A., De Graan, N.E., Gispen, W.H., and Wirtz, W.A. (1992) "Phosphatidic Acid Is a Specific Activator of Phosphatidylinositol-4-Phosphate Kinase," *J. Biol. Chem. 267,* 7207–7210.
37. Jenkins, G.H., Fisette, P.L., and Anderson, R.A. (1994) "Type I Phosphatidylinositol 4-Phosphate 5-Kinase Isoforms Are Specifically Stimulated by Phosphatidic Acid," *J. Biol. Chem. 269,* 11547–11554.
38. Shibata, M., Ohoka, T., Mizuno, S., and Suzuki, K. (1993) "Characterization of a 64-kD Protein Phosphorylated During Chemotactic Activation with IL-8 and fMLP of Human Polymorphonuclear Leukocytes. 1. Phosphorylation of a 64-kd Protein and Other Proteins," *J. Leukoc. Biol. 54,* 1–9.
39. Pike, M.C., Bruck, M.E., Arndt, C., and Lee, C.-S. (1990) "Chemoattractants Stimulate Phosphatidylinositol-4-Phosphate Kinase in Human Polymorphonuclear Leukocytes," *J. Bol. Chem. 265,* 1866–1873.

[Received July 27, 1994, and in final revised form March 14, 1995; Revision accepted March 21, 1995]

Diabetes and Proteolysis: Effects on Carnitine Palmitoyltransferase-I and Malonyl-CoA Binding

Khosrow Kashfi[1] Lauren Cagen and George A. Cook*

Department of Pharmacology, College of Medicine, University of Tennessee-Memphis, The Health Science Center, Memphis, Tennessee 38163

ABSTRACT: Malonyl-CoA binding and malonyl-CoA inhibition of carnitine palmitoyltransferase-I (CPT-I) were measured in hepatic mitochondria from normal and diabetic rats and in protease-treated mitochondria from fed rats to test the hypothesis that proteolysis represents a mechanism by which diabetes produces changes in the sensitivity of CPT-I to inhibition by malonyl-CoA. As in diabetes, protease treatment increased the apparent K_i values for malonyl-CoA. Palmitoyl-CoA greatly diminished malonyl-CoA specific binding in the mitochondrial system being studied, suggesting strong competition at the malonyl-CoA binding site. Proteolysis decreased capacity for specific binding of malonyl-CoA by 60–80%, but it had no effect on binding affinity. In contrast, the decreased specific binding of malonyl-CoA seen in the diabetic state is accompanied by increased binding affinity. Furthermore, observed K_d values differed from K_i values by a factor of 10 or more, suggesting that measured K_d and K_i may represent different ligand–protein complexes. These data suggest that alterations in inhibition of CPT-I by malonyl-CoA occurring in the diabetic state may involve mechanisms other than simple proteolytic removal of malonyl-CoA binding sites.

Lipids 30, 383–388 (1995).

Carnitine palmitoyltransferase (CPT; EC 2.3.1.21) is generally recognized as the most important regulatory enzyme in the hepatic mitochondrial fatty acid oxidation pathway. Two forms of CPT exist in mitochondria. CPT-I, located in the mitochondrial outer membrane, is inhibited by malonyl-CoA in concentrations that occur within the cytoplasm (1,2). CPT-II is located in the mitochondrial inner membrane and is insensitive to this inhibitor (1,2). The activity of CPT-I increases, and its sensitivity to inhibition by malonyl-CoA is diminished during fasting and diabetes (3–8). The most significant change that occurs with the onset of diabetes (7,8) or during a fasting period (9,10) is a 10-fold increase in the apparent K_i for malonyl-CoA.

The results of treating intact mitochondria and mitochondrial outer membranes with proteases led Murthy and Pande (1) to propose that the malonyl-CoA binding site of CPT-I faces the cytosol, while its catalytic site faces the intermembrane space. We recently evaluated the effects of proteases on the sensitivity of CPT-I to a variety of inhibitors (11,12). Sensitivity to most of these was not affected, suggesting that they act at the palmitoyl-CoA binding site facing the intermembrane space, but sensitivity to malonyl-CoA and 4-hydroxyphenylglyoxylate (HPG) was lost after protease treatment, suggesting that these act at a site facing the external medium. HPG is also the only inhibitor yet reported whose inhibitory potency is altered, like that of malonyl-CoA, by fasting and diabetes (13).

Since both protease treatment and diabetes reduce the sensitivity of CPT-I to inhibition by malonyl-CoA, and since proteolysis is enhanced in diabetes, we have previously proposed that proteolysis may be a mechanism by which diabetes alters the normal control of hepatic fatty acid oxidation (12). Similar proteolytic mechanisms have been found to occur in other metabolic pathways (14,15). The present study was conducted to compare the effect of diabetes with effects of protease treatment on both binding of [^{14}C]malonyl-CoA to intact mitochondria and malonyl-CoA inhibition of hepatic CPT-I since previous studies of malonyl-CoA binding to intact mitochondria (16,17) and mitochondrial outer membranes (18,19) have linked malonyl-CoA binding closely to malonyl-CoA inhibition of CPT-I.

EXPERIMENTAL PROCEDURES

Animals. Male Sprague-Dawley rats (200–250 g) obtained from Harlan Industries (Indianapolis, IN) were fed on Purina rat chow (Ralston Purina Co., Richmond, IN) and water *ad libitum.* On the day of the experiment, rats were killed by decapitation, and livers were removed rapidly for preparation of mitochondria. Ketotic diabetic rats used in some experiments were produced by injecting rats with 150 mg of streptozotocin/kg of body weight into the tail vein (20) and were used 48 h after the injection. This high dose of streptozotocin

[1]Current address: Department of Medicine, Division of Digestive Diseases, The New York Hospital-Cornell Medical Center, 525 East 68th St., New York, NY 10021.

*To whom correspondence should be addressed: Dr. George A. Cook,Department of Pharmacology, University of Tennessee, 874 Union Ave., Memphis, TN 38163-0001.

Abbreviations: BSA, bovine serum albumin; CPT-I, carnitine palmitoyltransferase of the mitochondrial outer membrane; HPG, 4-hydroxyphenylglyoxylate.

Copyright © 1995 by AOCS Press

was used to produce rats that were highly ketotic [urine ketone bodies ≥80 mg/mL and urine glucose levels ≥2000 mg/mL using Multistix® (Miles, Inc. Elkhart, IN)].

Isolation and protease treatment of mitochondria. Intact mitochondria were isolated by the method of Johnson and Lardy (21), with the modifications previously published (9), except that the isolation medium contained 210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, and 10 mM Tris/HCl (pH 7.4). In some experiments, mitochondria (5 mg protein/mL) were incubated at 37°C in isolation medium with subtilisin BPN′ (5 μg/mL). After incubation (2 to 20 min), the proteolytic activity was quenched by addition of 0.2 vol of bovine serum albumin (BSA) followed by addition of 40 mL of ice-cold isolation medium. Mitochondria were subsequently sedimented by centrifugation at 5600 × *g* for 10 min and then resuspended in isolation medium to a concentration of 20 mg protein per mL. In some initial experiments, 0.2 mM phenylmethylsulfonyl urea was added to the buffer after quenching, but it was found that the washing procedure with BSA was sufficient to prevent further proteolysis of the mitochondria. Protein was determined by a biuret reaction (22). The effects of the proteases papain, trypsin, and subtilisin BPN′ to decrease activity and malonyl-CoA sensitivity of CPT-I have been shown previously to be caused by proteolytic action since heating the proteases at 100°C for 10 min before use or addition of specific protease inhibitors prevented alterations of CPT-I (11,12). Also, increased effects of proteases were associated with increased incubation time and increased protease concentration but were decreased not only by protease inhibitors, but by specific inhibitors of CPT-I and by addition of BSA (11,12). Furthermore, the use of a specific chromogenic protease assay has been useful in correlating protease activity with alterations in CPT-I (12).

CPT. The method of Bremer (4) was used, modified as reported previously (7). Each assay contained, in a total volume of 1 mL, 82 mM sucrose, 70 mM KCl, 70 mM imidazole, 1 μg of antimycin A, 1 mM EGTA, 2 mg BSA, 40 μM palmitoyl-CoA, and 0.5 mM L-carnitine (0.4 mCi/mmol of L-[methyl-^{3}H]carnitine).

Binding of [2-^{14}C]malonyl-CoA. Malonyl-CoA binding experiments were performed in 1.5-mL plastic stoppered centrifuge tubes. The binding cocktail for incubations at 0°C contained, in a total volume of 1 mL, 82 mM sucrose, 70 mM KCl, 70 mM imidazole, 1 μg of antimycin A, and 1 mM EGTA. The pH was adjusted to 7.0 using HCl and KCl, to maintain equal ionic strength, according to Ellis and Morrison (23). Albumin was omitted from the binding cocktail since it had been shown to influence malonyl-CoA binding (24). The concentration of [^{14}C]malonyl-CoA (51 mCi/mmol) was usually varied from 5 to 660 nM, although higher concentrations were used in some initial experiments to show saturation of malonyl-CoA binding sites (Fig. 1). Experiments were started by addition of mitochondria (1 mg protein per mL). Equilibrium was reached within 120 min at 0°C with all treatments (Fig. 2), and incubations were terminated by centrifugation for 2 min at 12,000 × *g* using an Eppendorf micro-centrifuge at 0–4°C. Supernatant solution was aspirated, and the walls of the tubes were dried with tissue paper. The mitochondrial pellets were solubilized with 0.5 mL of 10% Triton X-100 at 37°C and transferred to scintillation vials. Radioactivity in the pellet and supernate was measured by scintillation spectrometry. Nonspecific binding was estimated by inclusion of 250 nmol unlabeled malonyl-CoA in the incubation medium (4 μmol unlabeled malonyl-CoA were used when the concentration of labeled ligand exceeded 660 nM). Nonspecific binding was proportional to the con-

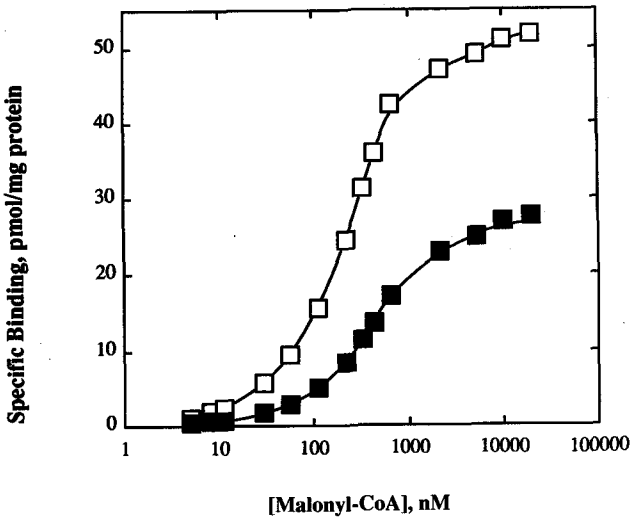


FIG. 1. Saturation binding curve represented as specific binding as a function of the log of malonyl-CoA concentration. Specific binding of [^{14}C]malonyl-CoA was measured at 0°C in control (□) and protease-treated (■) intact hepatic mitochondria as described in the Materials and Methods section.

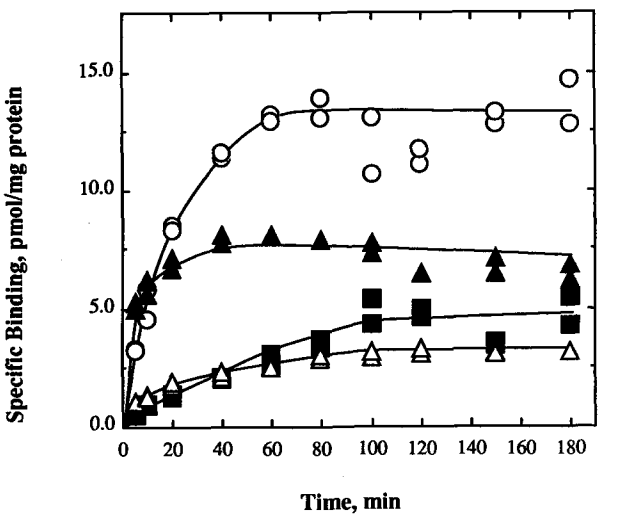


FIG. 2. Equilibrium time course for different mitochondrial treatments. Specific binding was measured as a function of time at 0°C in control (○), diabetic (▲), protease-treated (■), and control plus 5 μM palmitoyl-CoA (△) using intact hepatic mitochondria that had been incubated with [^{14}C]malonyl-CoA as described in the Materials and Methods section.

centration of labeled malonyl-CoA and was nonsaturable (data not shown); specific binding was calculated by subtracting nonspecific binding from total binding at each concentration of ligand.

Analysis of [2-^{14}C]malonyl-CoA binding data. The binding data were plotted according to Scatchard (25). This plot gave a curve which was upward concave in shape. Two possible biological interpretations of these data are possible: (i) multiple binding sites exist with unchanging and different affinities; and (ii) negative cooperativity exists, such that the affinity of the overall binding population decreases with increasing occupancy of the binding sites (26). In order to distinguish between these two possibilities, "infinite dilution" experiments were carried out according to De Meyts *et al.* (27). Mitochondria (1 mg protein per mL) were incubated with [2-^{14}C]malonyl-CoA at 0°C for 120 min. After centrifugation at 12,000 × *g*, the mitochondrial pellet was resuspended in 1 mL of binding cocktail without added malonyl-CoA, and 100 µL was removed for determination of "zero time" binding. Additional 100 µL aliquots were diluted in 10 mL of cocktail at 0°C with or without a 100-fold excess of unlabeled malonyl-CoA. At various times, the suspensions were centrifuged, and bound radioactivity was determined as described above. From a plot of malonyl-CoA bound vs. time elapsed after dilution (Fig. 3), it was concluded that negative cooperativity did not exist since the presence of an excess of unlabeled ligand did not affect the rate of dissociation of the labeled ligand. Infinite dilution experiments were also carried out for nonspecific binding. Nonspecific binding represented only a small fraction of the total radioactivity bound, and dissociation was much more rapid so that the dissociation rates observed in the infinite dilution experiments were not affected by nonspecific binding.

After establishing that the Scatchard plots represented multiple binding sites, estimates of binding parameters were obtained using the computer programs EBDA (28) and LIGAND (29,30). The analysis of the data fitted the two-site models ($P < 0.05$) better than the one-site ($P \geq 0.1$) model when residual sums of squares were compared. Hence, all parameters reported here are for a two-site model. These data clearly suggest that malonyl-CoA binds to more than one site in mitochondrial membranes, although binding data alone cannot indicate the nature of the proteins involved.

Materials. Palmitoyl-CoA, imidazole, BSA, malonyl-CoA, EGTA, Subtilisin BPN′ (Catalogue # P 4789), and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO). L-[methyl-^{3}H]Carnitine hydrochloride and [2-^{14}C]malonyl-CoA were obtained from Amersham Corporation (Arlington Heights, IL).

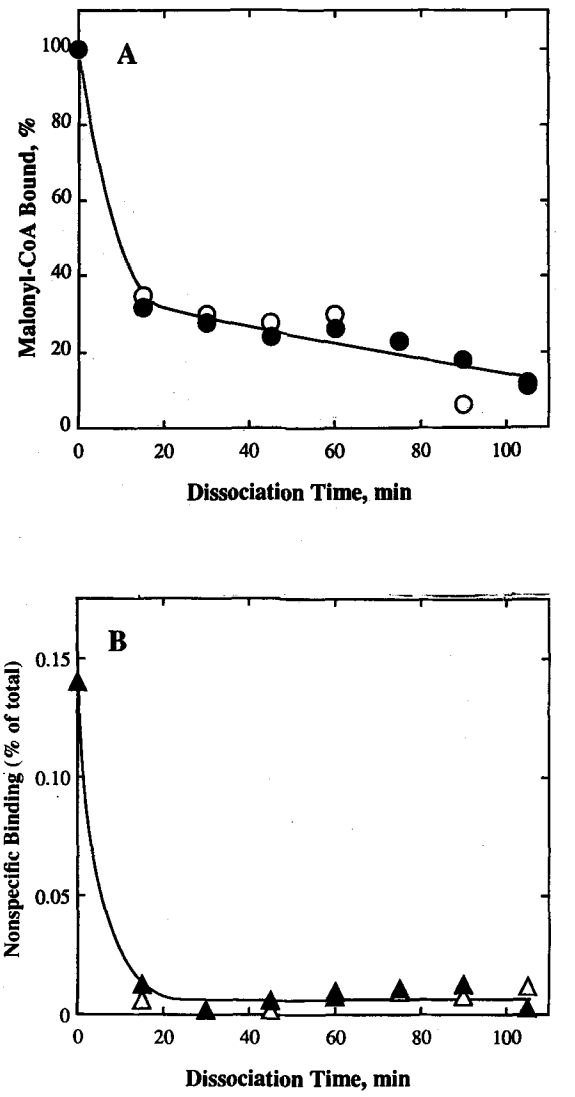


FIG. 3. Dissociation of bound [^{14}C]malonyl-CoA from mitochondria. A. [^{14}C]Malonyl-CoA and intact hepatic mitochondria were first incubated at 0°C for 120 min to allow binding equilibrium to be reached, then mitochondria were centrifuged and diluted with (●) or without (○) a 100-fold excess of unlabeled malonyl-CoA to allow dissociation of malonyl-CoA. Labeled malonyl-CoA remaining bound was measured at the times indicated. B. Dissociation of nonspecific binding. Conditions were identical with A; ▲, with; △, without.

RESULTS

Effects of protease treatment on the apparent K_i for malonyl-CoA. We previously presented data indicating that limited proteolysis of the intact hepatic mitochondria resulted in altered kinetic properties of CPT-I as indicated by changes in the shapes of Dixon (31) plots, suggesting that positive cooperativity was lost as a result of protease treatment (12). Therefore, we wanted to find out what effect proteolysis would have on the apparent K_i for malonyl-CoA after exposure of the intact mitochondria to protease for different time periods. We used the low concentration end of the Dixon plots (1–5 µM) to estimate apparent K_i values for malonyl-CoA. At these concentrations, graphs were linear and a direct estimation of apparent K_i could be made. The calculated apparent K_i values for malonyl-CoA are presented in Figure 4. The data indicate that exposure of intact hepatic mitochondria to the protease greatly increases the apparent K_i values for mal-

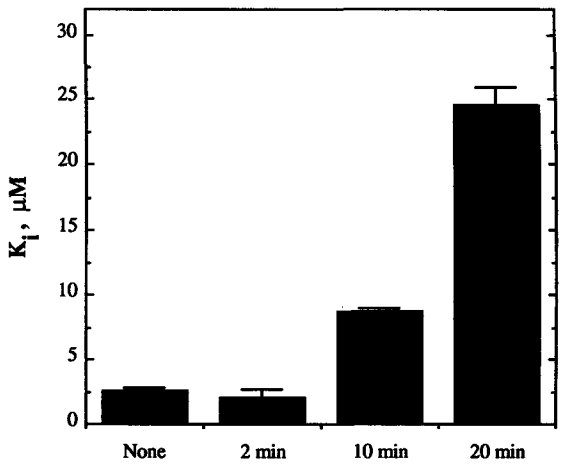


FIG. 4. Effects of proteolysis on the apparent K_i of carnitine palmitoyltransferase-I for malonyl-CoA. K_i values were determined from Dixon plots (Ref. 31) that were constructed for control and protease-treated intact mitochondria using 5 concentrations of malonyl-CoA at each of three concentrations of palmitoyl-CoA at the conditions indicated in the figure. Results are means ± SEM for three different preparations of mitochondria.

onyl-CoA in a manner dependent on the extent of proteolysis and similar to effects seen with the onset of diabetes (6–8).

Effect of protease treatment on specific binding of [2-^{14}C]malonyl-CoA to hepatic mitochondria. Since protease treatment and diabetes both increase the apparent K_i values for malonyl-CoA, we wanted to determine whether proteolysis would alter malonyl-CoA binding to intact hepatic mitochondria to suggest a mechanism for the effects of diabetes. The time course of specific association of [2-^{14}C]malonyl-CoA with intact rat hepatic mitochondria at 0°C, with or without prior treatment with protease for 20 min, is shown in Figure 2. Equilibrium was achieved within 120 min. Treatment with protease for 20 min reduced the extent of [2-^{14}C]malonyl-CoA binding (Figs. 1 and 5). Analysis of the binding data for control mitochondria by LIGAND was consistent with a two-site model (Table 1). High affinity sites (K_d = 63 nM) represented 26% of the total binding capacity; the remainder reflected low affinity (K_d = 257 nM) sites. Dissociation of bound [2-^{14}C]malonyl-CoA was biphasic whether net dissociation was initiated by dilution or by addition of excess unlabeled ligand (Fig. 3). This is consistent with a model composed of two independent classes of binding site. Under conditions of the experiment, about one-third of the ligand was bound to slowly dissociating sites. Treatment with protease removed 79% of the high-affinity sites and 62% of the low affinity sites with no significant change in the apparent affinity of the surviving sites.

Binding of [2-^{14}C]malonyl-CoA to mitochondria from diabetic rats. Similar to the effects of protease treatment, streptozotocin-induced diabetes reduced the number of high- and low-affinity binding sites (by 58 and 56%, respectively) for [2-^{14}C]malonyl-CoA in hepatic mitochondria (Figs. 5 and 6; Table 1); however, in contrast to protease treatment, the apparent affinities of the remaining sites were significantly enhanced in hepatic mitochondria from diabetic rats. Palmitoyl-CoA was added in some experiments to show the effects of competition of malonyl-CoA and palmitoyl-CoA, indicating that malonyl-CoA is binding specifically and competitively to an acyl-CoA binding enzyme. Addition of palmitoyl-CoA increased both dissociation constants for malonyl-CoA, suggesting that both sites are related to CPT function.

TABLE 1
Malonyl-CoA Binding Parameters for Binding to Intact Hepatic Mitochondria[a]

Treatment	K_{d1} (nM)	K_{d2} (nM)	N_1 (pmol/mg)	N_2 (pmol/mg)
Control	63 ± 13	257 ± 20	19 ± 3	52 ± 5
Protease	47 ± 4	366 ± 50	4 ± 0.2[b]	20 ± 4[b]
Diabetic	18 ± 2[b]	170 ± 14[b]	8 ± 0.6[b]	29 ± 4[b]
Control + 5 μM palmitoyl-CoA	102 ± 1[b]	2321 ± 124[b]	6 ± 4	91 ± 12

[a]Malonyl-CoA binding was measured under the conditions described in the Materials and Methods section. Protease treatment was carried out using the protease subtilisin BPN' at 5 μg/mL for 20 min. Diabetic rats were produced by streptozotocin injection and were ketotic. N_1 and N_2 represent numbers of high- and low-affinity binding sites for malonyl-CoA, respectively. Results are mean ± SEM for three different preparations of mitochondria.
[b]Significantly different from control values ($P < 0.05$).

DISCUSSION

The effects of proteolysis on the apparent K_i values for malonyl-CoA raised the possibility that endogenous proteolysis of hepatic mitochondrial CPT-I might be a mechanism for changing the apparent K_i values for malonyl-CoA in such states as fasting and diabetes, where proteolysis is most

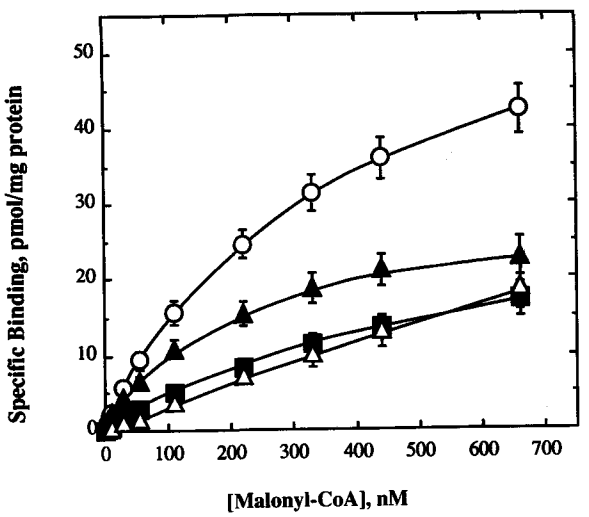


FIG. 5. Specific binding of malonyl-CoA in normal, diabetic, and protease-treated mitochondria. Specific binding of [^{14}C]malonyl-CoA was measured at 0°C in control (○), diabetic (▲), protease-treated (□), and control mitochondria plus 5 μM palmitoyl-CoA (△) as described in the Materials and Methods section. Results are means ± SEM for three different preparations of mitochondria.

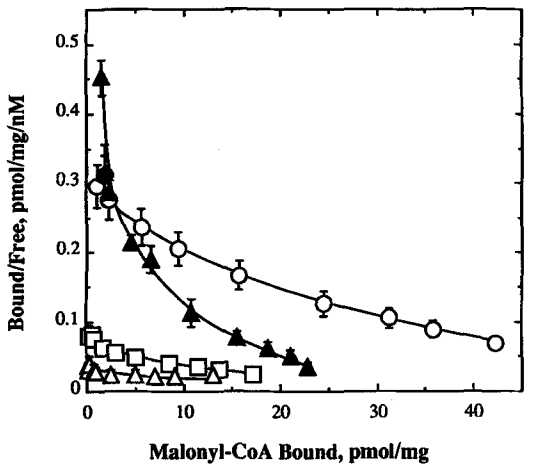


FIG. 6. Scatchard analysis of the binding data. Scatchard analysis of the binding data was carried out as described in the Materials and Methods section. The symbols refer to control (○), diabetic (▲), protease-treated (□), and control in the presence of 5 μM palmitoyl-CoA (△). Results are means ± SEM for three different preparations of mitochondria.

prevalent. There are a number of reports which indicate that isovalerylcarnitine, a product of L-leucine catabolism, activates the endogenous protease calpain II and modulates the effects of calpstatin, an endogenous inhibitor of calpain II, suggesting a possible mechanism for initiation of proteolysis during starvation or diabetes (14,15). The binding data we obtained from diabetic animals showing a significant decrease in the number of both the high- and low-affinity binding sites is comparable to the effects of protease treatment of intact normal mitochondria and further suggests that proteolysis may be a mechanism for changes observed during diabetes. However, the change in binding affinity with diabetes, but not with proteolysis, suggests that the proteolytic removal of malonyl-CoA binding sites is not sufficient to account for all the effects of diabetes on CPT-I. The increasing affinity for malonyl-CoA during diabetes is paradoxical. It suggests the binding of malonyl-CoA during the diabetic state may be unrelated or somehow dissociated from malonyl-CoA inhibition. This further suggests that the effects of diabetes are more complex than previously expected. We have recently found that diabetes increases CPT-I mRNA 15-fold, suggesting greatly increased rates of transcription (data not shown).

Proteolytic treatment of intact hepatic mitochondria increased the apparent K_i for malonyl-CoA as a function of treatment time; however, there were no changes in the dissociation constants for malonyl-CoA binding to intact hepatic mitochondria. This suggests that the apparent K_i for malonyl-CoA and the K_d values for malonyl-CoA binding do not represent the same dissociation constants. One would expect these two values to be identical since the K_i value for a competitive inhibitor is the equilibrium constant for the dissociation of the enzyme-inhibitor complex. The only effect of proteolysis is to decrease the number of binding sites. This suggests that the K_d values measured here relate to the enzyme remaining after protease treatment and that the protease eliminates malonyl-CoA binding completely without altering binding characteristics. Since the K_i is changed but the K_d is not, it is possible that the K_i is for an association involving malonyl-CoA, CPT-I that is unaltered by protease treatment, and an auxillary protein binding malonyl-CoA that is digested by the protease. Recent data have indicated that it may be possible to discriminate two components of liver CPT-I, a catalytic component and a regulatory component that binds malonyl-CoA (32–34). If this hypothesis is correct, then our present data suggests that K_{d1} and K_{d2} represent dissociation constants of separate complexes formed by malonyl-CoA binding to the binding protein (the regulatory component) and to the catalytic component, while K_i represents a more complex dissociation constant of a ternary complex comprising malonyl-CoA, the regulatory binding component, and the catalytic component of CPT-I.

In light of the known effects of diabetes to decrease malonyl-CoA inhibition by increasing the apparent K_i value for malonyl-CoA (7,8), the binding parameters measured for [2-^{14}C]malonyl-CoA to the hepatic mitochondria from diabetic animals were surprising. That numbers of both the high- and the low-affinity binding sites were significantly reduced is easily understood. However, the fact that both K_d values decreased during diabetes, which is exactly opposite to the effect on apparent K_i values, again suggests that K_i and K_d values do not represent the same dissociation constants for CPT-I. Diabetes may act to dissociate malonyl-CoA binding and malonyl-CoA inhibition of CPT-I. Using isolated mitochondrial outer membranes, it was reported that diabetes increased the number of high-affinity binding sites while increasing the high-affinity dissociation constant and decreasing both the number and affinity of the low-affinity sites (18). Our data are contrary to these findings, possibly because: (i) the protocols that these authors used (18) included BSA, which interferes with malonyl-CoA binding (24); (ii) binding was conducted for only 20 min, which our data indicates is too short a time period to reach binding equilibrium; (iii) their binding medium was at pH 6.8, a condition which favors tighter binding (24); (iv) the membranes were washed twice with their binding medium, and significant amounts of malonyl-CoA could have been lost in washing; and (v) no corrections were made for nonspecific binding.

The primary structure of CPT-I has recently been deduced from the sequence of a cDNA which suggests that CPT-I has a single membrane-spanning region (35). When our previous work on proteolytic effects on CPT-I (11,12) and that of Murthy and Pande (1) are considered when examining the deduced sequence, it becomes apparent that most of the CPT-I protein, containing the active site, must lie inside the mitochondrial outer membrane but that approximately 100 amino acid residues at the N-terminal end lies outside that membrane in the cytosolic space. It is this N-terminal region which may be susceptible to attack by endogenous proteases or to proteases added *in vitro*, and when this region is removed or damaged by proteases, CPT-I loses its ability to be inhibited by malonyl-CoA. Alternatively, proteases may act on a puta-

tive regulatory protein. Recent work on the kinetics of CPT-I has shown that this enzyme has two independent binding sites for inhibitors, with malonyl-CoA preferring a noncatalytic site located outside the mitochondrial outer membrane (36). Whether this site resides on the catalytic protein or on a regulatory subunit remains to be resolved; however, the data presented here suggest that the changes in malonyl-CoA sensitivity of CPT-I that occur in diabetic animals may be due to mechanisms other than simple proteolytic removal of malonyl-CoA binding sites.

ACKNOWLEDGMENTS

This work was supported by grant HL-40929 from the National Institutes of Health (U.S. Public Health Service). Additional support was received through a Grant-in-Aid from the American Heart Association, Tennessee Affiliate.

REFERENCES

1. Murthy, M.S.R., and Pande, S.V. (1987) *Proc. Natl. Acad. Sci., U.S.A. 84,* 378–382.
2. McGarry, J.D., and Foster, D.W. (1980) *Annu. Rev. Biochem. 49,* 395–420.
3. Cook, G.A., Otto, D.A., and Cornell, N.W. (1980) *Biochem. J. 192,* 955–958.
4. Bremer, J. (1981) *Biochim. Biophys. Acta 665,* 628–631.
5. Saggerson, E.D., and Carpenter, C.A. (1981) *FEBS Lett. 129,* 225–228.
6. Gamble, M.S., and Cook, G.A. (1985) *J. Biol. Chem. 260,* 9516–9519.
7. Cook, G.A., and Gamble, M.S. (1987) *J. Biol. Chem. 262,* 2050–2055.
8. Kashfi, K., Weakley, L.J., and Cook, G.A. (1988) *Biochem. Soc. Trans. 16,* 1010–1011.
9. Cook, G.A. (1984) *J. Biol. Chem. 259,* 12030–12033.
10. Cook, G.A., Kashfi, K., and Weakley, L.J. (1988) *FASEB. J. 2,* A1035.
11. Kashfi, K., and Cook, G.A. (1991) *Biochem. Biophy. Res. Commun. 178,* 600–605.
12. Kashfi, K., and Cook, G.A. (1992) *Biochem. J. 282,* 909–914.
13. Stephens, T.W., and Harris, R.A. (1987) *Biochem. J. 243,* 405–412.
14. Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Siliprandi, N., and Horecker, B.L. (1987) *Biochem. Biophy. Res. Commun. 148,* 1189–1195.
15. Pontremoli, S., Melloni, E., Viotti, P.L., Michetti, M., Di Lisa, F., and Siliprandi, N. (1990) *Biochem. Biophy. Res. Commun. 167,* 373–380.
16. Mills, S.E., Foster, D.W., and McGarry, J.D. (1983) *Biochem. J. 214,* 83–91.
17. Bird, M.I., and Saggerson, E.D. (1984) *Biochem. J. 222,* 639–647.
18. Ghadiminejad, I., and Saggerson, E.D. (1991) *Biochem. J. 277,* 611–617.
19. Kolodziej, M.P., and Zammit, V.A. (1990) *Biochem. J. 267,* 85–90.
20. Schein, P.S., Alberti, K.G.M.M., and Williamson, D.H. (1971) *Endocrinology 89,* 827–834.
21. Johnson, D., and Lardy, H. (1967) *Methods Enzymol. 10,* 94–96.
22. Layne, E. (1957) *Methods Enzymol. 3,* 450–451.
23. Ellis, K.J., and Morrison, J.F. (1982) *Methods Enzymol. 87,* 405–427.
24. Lund, H., and Woldegiorgis, G. (1986) *Biochim. Biophys. Acta 878,* 243–249.
25. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51,* 660–672.
26. Limbird, L.E. (1986) *Cell Surface Receptors: A Short Course on Theory and Methods,* pp. 51–96, Martinus Nijhoff, Boston.
27. De Meyts, P., Bianco, A.R., and Roth, J. (1976) *J. Biol. Chem. 251,* 1077–1888.
28. McPherson, G.A. (1985) in *A Collection of Radioligand Binding Analysis Programs,* pp. 1–127, BIOSOFT®, Cambridge.
29. Munson, P.J., and Rodbard, D. (1980) *Anal. Biochem. 107,* 220–239.
30. Munson, P.J. (1983) *Methods Enzymol. 92,* 543–576.
31. Dixon, M. (1953) *Biochem J. 55,* 170–171.
32. Ghadiminejad, I., and Saggerson, E.D. (1990) *FEBS Lett. 269,* 406–408.
33. Ghadiminejad, I., and Saggerson, E.D. (1991) *Biochim. Biophys. Acta 1083,* 166–172.
34. Woldegiorgis, G., Fibich, B., Contreras, L., and Shrago, E. (1992) *Arch. Biochem. Biophys. 295,* 345–351.
35. Esser, V., Britton, C.H., Weis, B.C., Foster, D.W., and McGarry, J.D. (1993) *J. Biol. Chem. 268,* 5817–5822.
36. Cook, G.A., Mynatt, R.L., and Kashfi, K. (1994) *J. Biol. Chem. 269,* 8803–8807.

[Received September 30, 1994, and in revised form February 8, 1995; Revision accepted February 13, 1995]

The Effect of Two Synthetic Phospholipids on Cell Proliferation and Phosphatidylcholine Biosynthesis in Madin-Darby Canine Kidney Cells

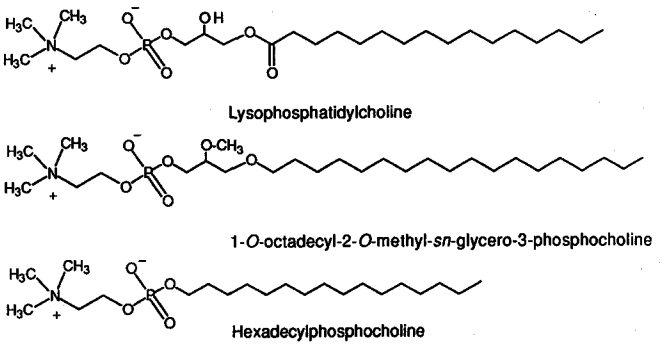
Thomas Wieder, Anne Haase, Christoph C. Geilen* and Constantin E. Orfanos

Haut- und Poliklinik, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, D-12200 Berlin, Germany

ABSTRACT: The concentration-dependent effects of two different synthetic phospholipids on cell proliferation and phosphatidylcholine biosynthesis were compared in Madin-Darby canine kidney (MDCK) cells. The alkyllysophospholipid 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine and the alkylphosphocholine, hexadecylphosphocholine, inhibited cell proliferation with half-inhibitory concentrations (IC_{50}) of 75 and 135 μmol/L, respectively. The agents also inhibited phosphatidylcholine biosynthesis in confluent and proliferating MDCK cells. The IC_{50} of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine was 40 μmol/L in confluent cells and 20 μmol/L in proliferating cells, whereas the IC_{50} of hexadecylphosphocholine was higher in both experimental systems (67 μmol/L in confluent cells and 40 μmol/L in proliferating cells). Further experiments revealed that the effect of both agents on phosphatidylcholine biosynthesis was reversible and that the inhibition was mediated by translocation of the rate-limiting enzyme of this pathway, CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15), from membranes to the cytosol, where it is inactive. The present findings suggest that the inhibition of phosphatidylcholine biosynthesis by both synthetic phospholipids might be related, in part, to their antiproliferative effects.

Lipids 30, 389–393 (1995).

Alkyllysophospholipids have been shown to possess antineoplastic properties *in vitro* and *in vivo* (for a review, see Ref. 1). A number of hypotheses have been brought forth to explain their mechanism(s) of action (2,3). One proposed mechanism is the perturbation of reacylation of lysophospholipids that is mediated by lysophosphatidylcholine:acyl-CoA transferase (4). It was shown that the alkyllysophospholipid 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3) inhibits the incorporation of unsaturated fatty acids into phosphatidylcholine in HL60 cells (4). However, in other studies a correlation between the inhibition of cell proliferation and the effect on incorporation of fatty acids into phospholipids could not be demonstrated (5,6).



SCHEME 1

Alkylphosphocholines, another class of synthetic phospholipids, are effective antineoplastic agents which have been used as anticancer drugs against skin metastases of human breast carcinoma (7). We have previously shown that alkylphosphocholines inhibit the biosynthesis of phosphatidylcholine by disturbing the translocation process of the rate-limiting enzyme, CTP: phosphocholine cytidylyltransferase (EC 2.7.7.15), and that this inhibition correlates with the antiproliferative effect of these agents (8–10).

In the present study, we compared the effects of ET-18-OCH_3 and hexadecylphosphocholine (HePC) (Scheme 1) on cell proliferation and phosphatidylcholine biosynthesis in Madin-Darby canine kidney (MDCK) cells. ET-18-OCH_3 inhibited cell proliferation and phosphatidylcholine biosynthesis, and the inhibitory effects of ET-18-OCH_3 were stronger as compared with the effects of HePC. However, the cytotoxicity of ET-18-OCH_3 in our experimental system was also quite high. From our results, we conclude that the influence of HePC on phosphatidylcholine biosynthesis is more specific because inhibition of phosphatidylcholine biosynthesis by HePC clearly preceded cell death.

EXPERIMENTAL PROCEDURES

Materials. ET-18-OCH_3 was obtained from Novabiochem (Bad Soden, Germany), and HePC was synthesized as de-

*To whom correspondence should be addressed at Haut- und Poliklinik, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany.

Abbreviations: BCA, bicinchoninic acid; ET-18 $0CH_3$, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; HePC, hexadecylphosphocholine; HPTLC, high-performance thin-layer chromatography; IC_{50}, half-inhibitory concentration; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Copyright © 1995 by AOCS Press

scribed previously (11). [*Methyl*-^{3}H]choline chloride (2.8–3.1 TBq/mmol) was from Amersham (Braunschweig, Germany). Silica gel 60 high-performance thin-layer chromatography (HPTLC) plates, solvents, and reagents (reagent grade) were purchased from E. Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany). The bicinchoninic acid (BCA) kit for protein determination was obtained from Pierce (Weiskirchen, Germany). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), choline, phosphocholine, and CDP-choline were from Sigma (München, Germany). For quantification of radioactivity a Berthold (Wildbad, Germany) LB 2821 HR thin-layer chromatography scanner was used.

Cell culture. MDCK cells were grown in plastic culture dishes (Nunc, Denmark) containing Dulbecco's minimal essential medium supplemented with 10% calf serum, 0.56 g/L L-glutamine, 100,000 IU penicillin, and 0.1 g/L streptomycin. Media and culture reagents were obtained from Gibco (Berlin, Germany), and penicillin and streptomycin from Boehringer (Mannheim, Germany). Confluent cells were subcultured every three days after detaching the cells with a 0.1% trypsin/0.02% EDTA solution.

Cytotoxicity assay. Cytotoxicity of the different synthetic phospholipids to MDCK cells was estimated according to Culvenor *et al.* (12) by measurement of the alkaline phosphatase activity of the cells. Confluent cells were incubated for 6 h with medium containing different concentrations of ET-18-OCH_3 and HePC or no additional supplements as controls. The cells were centrifuged at 1000 × *g* for 10 min, and the supernatant was discarded. The reaction mixture [containing 20% diethanolamine, 2 µmol/L $MgCl_2$, 2 µmol/L *p*-nitrophenylphosphate, and 10% Triton X-100 (Aldrich, Steinheim, Germany)] was added. After 1 h at 37°C, the reaction was stopped with 0.5 mol/L NaOH. The amount of *p*-nitrophenolate was determined photometrically at 410 nm.

Cell proliferation assay. Proliferating MDCK cells (approximately 20,000 cells/cm^2) were incubated with medium containing different concentrations of ET-18-OCH_3 and HePC or no additional supplements as controls. After 16 h, the proliferation rate was determined by the crystal violet assay as described (13) and compared with that of control cells. Cells were fixed by treatment with 1% glutaraldehyde for 15 min, stained for 30 min in 0.1% crystal violet solution, then washed with deionized water for 15 min. Crystal violet absorbed by cell nuclei was solubilized with 0.2% Triton X-100, and the amount released was quantitated photometrically at 590 nm.

Measurement of phosphatidylcholine biosynthesis. Confluent (72 h after plating) and proliferating (24 h after plating) MDCK cells were incubated for 6 h with pulse medium (1 µCi/mL [*methyl*-^{3}H]-choline) which was supplemented with ET-18-OCH_3 or HePC in concentrations of 0, 10, 50, 100, or 200 µmol/L. After incubation, cells were washed two times with ice-cold phosphate-buffered saline (PBS) (pH 7.2) and harvested with a cell lifter (Costar, Cambridge, MA), followed by modified lipid extraction according to Bligh and Dyer (14). After lyophilization of the cells, 50 µL methanol, 25 µL chloroform, and 20 µL water were added. Samples were stirred for 2 min on a vortex mixer and centrifuged at 13,000 × *g* for 10 min. Phase separation was accomplished by addition of 25 µL chloroform and 25 µL water. The suspension and centrifugation steps were repeated. Then 10 µL of the chloroform phases were taken for scintillation counting, and 15 µL were applied to silica gel 60 HPTLC plates using a HPTLC applicator (Linomat III; Camag, Berlin, Germany). Lipids were separated according to Touchstone *et al.* (15) using the solvent chloroform/methanol/triethylamine/water (30:35:34:8, by vol). Radioactivity was quantified by radioscanning. Phospholipids were identified by calibrating the scanner with known standards. Approximately 95% of the radioactivity co-chromatographed with the phosphatidylcholine standard.

For determination of phosphatidylcholine biosynthesis under stimulating conditions, cells were simultaneously treated with 5 nmol/L TPA plus 50 µmol/L ET-18-OCH_3, and 5 nmol/L TPA plus 50 µmol/L HePC.

Reversibility of the inhibitory effect of both synthetic phospholipids was investigated using a different protocol. In these experiments, cells were preincubated for 6 h with medium, containing 50 µmol/L ET-18-OCH_3 or 50 µmol/L HePC. The cells were washed two times with warmed culture medium, and labeling was initiated by adding pulse medium (1 µCi/mL [*methyl*-^{3}H]choline) without additional supplements. After 1, 2, and 6 h, cells were collected, and incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine was measured as described previously. Phosphatidylcholine biosynthesis under control conditions was determined using cells which had been preincubated and labeled with medium containing no additional supplements. Furthermore, phosphatidylcholine biosynthesis under inhibitory conditions was measured using cells which had been preincubated and labeled with medium containing 50 µmol/L ET-18-OCH_3 or 50 µmol/L HePC.

Digitonin-mediated release of cytidylyltransferase from MDCK cells. The release of cytidylyltransferase from MDCK cells was measured according to Pelech *et al.* (16). Cells were incubated for 4 h in 35-mm dishes with medium containing the appropriate supplements (as described for the labeling experiments), then washed twice with 1 mL ice-cold PBS. Digitonin-mediated release of cytidylyltransferase was performed as described earlier (17). The distribution of cytidylyltransferase activity between cytosol and membranes was calculated from the data for each dish.

CTP:phosphocholine cytidylyltransferase assay. The enzyme activity was measured by a modified method of Sohal and Cornell (18) as described (17), using liposomes (400 µmol/L phosphatidylcholine and 400 µmol/L oleic acid) and 10 µL of enzyme preparation in a final volume of 55 µL. One unit of enzyme activity is defined as 1 nmol of CDP-choline formed per minute.

Other procedures. Cellular protein was determined in each sample of the radiolabeling experiments by the BCA assay (19), using bovine serum albumin as a standard. Statistical comparisons were made in these studies with Student's *t*-test.

RESULTS

We first determined the cytotoxicity of ET-18-OCH_3 and HePC in MDCK cells. As shown in Figure 1, a dose-dependent increase of the cytotoxic effect was seen for both substances. However, at concentrations of 50 µmol/L or less the synthetic phospholipids did not show any cytotoxicity (viability >95%).

Cell proliferation was also inhibited by ET-18-OCH_3 and HePC. Whereas ET-18-OCH_3 had a half-inhibitory concentration (IC_{50}) of 75 µmol/L, HePC was less inhibitory with an IC_{50} of 135 µmol/L (Fig. 2). Nevertheless, the antiproliferative effect of HePC seemed to be more specific because the cells were still viable when cell proliferation was totally abolished.

The incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine in MDCK cells treated with different concentrations of ET-18-OCH_3 was investigated and compared with the effect of the alkylphosphocholine, HePC. In confluent, nonproliferating cells, the IC_{50} of ET-18-OCH_3 was 40 µmol/L compared with an IC_{50} of HePC of 67 µmol/L (Fig. 2). In a second set of experiments, we used proliferating cells 24 h after plating the cells at a density of 20,000 cells/cm^2. Under these conditions, the cells had a doubling-time of about 24 h (data not shown). Proliferating cells appeared to be more sensitive than confluent cells and revealed IC_{50} levels of 20 µmol/L (ET-18-OCH_3) and 40 µmol/L (HePC) when incorporation of [*methyl*-^{3}H]-choline into phosphatidylcholine was measured (Fig. 3).

Recently, we found that phorbol ester-stimulated phosphatidylcholine biosynthesis is antagonized by simultaneous treatment with HePC (8). Therefore, we compared the effects of 50 µmol/L ET-18-OCH_3 and 50 µmol/L HePC on phorbol ester-stimulated phosphatidylcholine biosynthesis in MDCK cells. Phorbol ester treatment stimulated [*methyl*-^{3}H]choline incorporation into phosphatidylcholine by 600 ± 50% as compared with nontreated controls. This stimulation of phosphatidylcholine biosynthesis was completely antagonized by both synthetic phospholipids. Incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine was 25 ± 5% and 50 ± 6% as compared with controls when cells were incubated with TPA plus ET-18-OCH_3 or with TPA plus HePC, respectively.

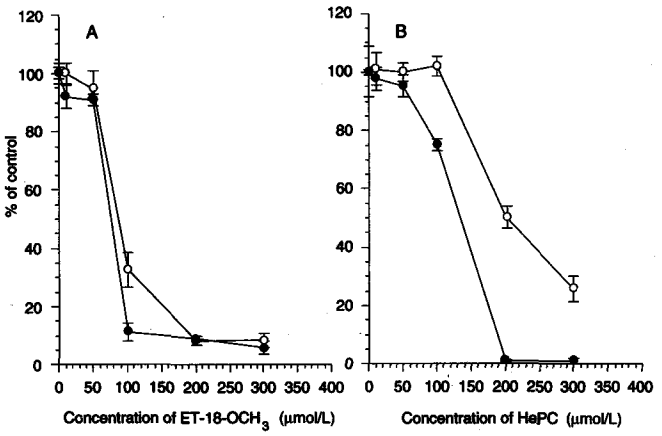


FIG. 2. Concentration-dependent effects of ET-18-OCH_3 and HePC on phosphatidylcholine biosynthesis in confluent MDCK cells. After 72 h, confluent MDCK cells were incubated for 6 h with pulse medium containing 2.5 µCi/mL [*methyl*-^{3}H]choline and different concentrations of ET-18-OCH_3 (A) and HePC (B). After 6 h of labeling, the incorporation of radioactivity into phosphatidylcholine was determined as described in the Experimental Procedures section. Values are given in % of controls ± SD (n = 3). The incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine in control cells was 480 ± 33 dpm/µg cellular protein. Abbreviations as in Figure 1. IC_{50}, half-inhibitory concentration.

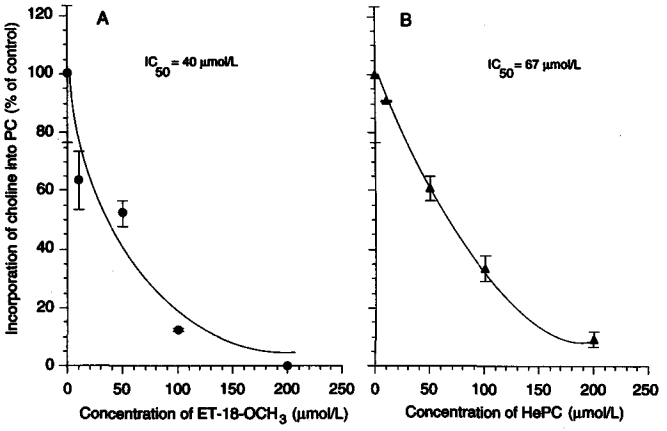


FIG. 1. Cytotoxicity and antiproliferative effect of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3) and hexadecylphosphocholine (HePC). After incubation of confluent Madin-Darby canine kidney (MDCK) cells for 6 h with the indicated concentrations of ET-18-OCH_3 (A) or HePC (B), the cytotoxicity (○) was measured as described in the Experimental Procedures section. Proliferating MDCK cells were incubated with different concentrations of ET-18-OCH_3 (A) or HePC (B). After 16 h, the proliferation rate (●) was determined by crystal violet assay as described in the Experimental Procedures section. Values are given in % of controls ± SD (n = 4).

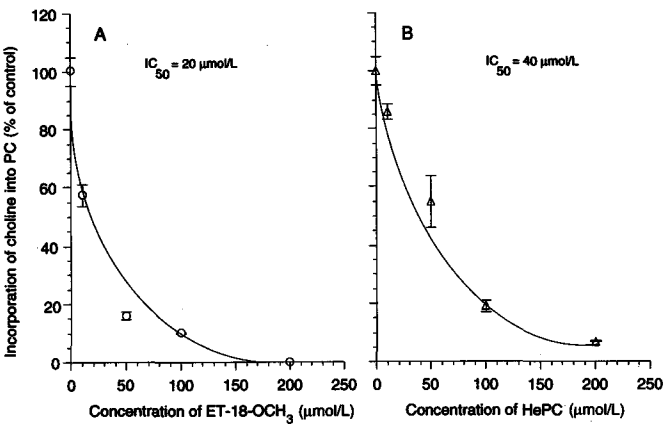


FIG. 3. Concentration-dependent effects of ET-18-OCH_3 and HePC on phosphatidylcholine biosynthesis in proliferating MDCK cells. After 24 h, proliferating MDCK cells were incubated for 6 h with pulse medium containing 2.5 µCi/mL [*methyl*-^{3}H]choline and different concentrations of ET-18-OCH_3 (A) and HePC (B). After 6 h of labeling, the incorporation of radioactivity into phosphatidylcholine was determined as described in the Experimental Procedures section. Values are given in % of controls ± SD (n = 3). The incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine in control cells was 480 ± 33 dpm/µg cellular protein. Abbreviations as in Figure 2.

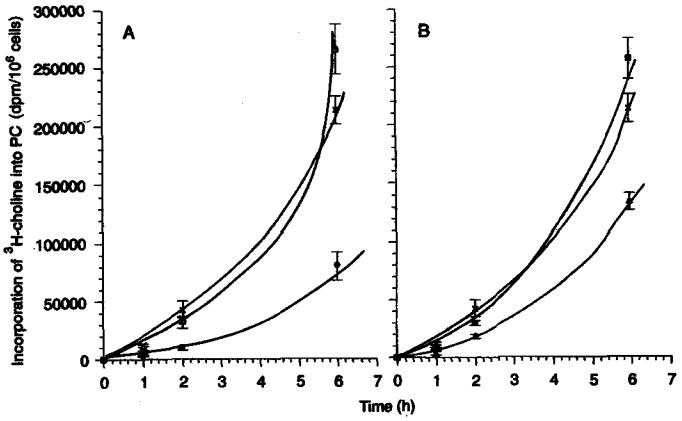


FIG. 4. Reversibility of the effect of ET-18-OCH_3 and HePC on phosphatidylcholine biosynthesis. Cells were preincubated with 50 μmol/L ET-18-OCH_3 (□, A), 50 μmol/L HePC (■, B) or without supplements (×, A, B) for 6 h. Then the medium was replaced by pulse medium containing 1 μCi/mL [*methyl*-^{3}H]choline. The cells were incubated for 1 h, 2 h or 6 h. Incorporation of radioactivity into phosphatidylcholine was determined as described in the Experimental Procedures section. Furthermore, phosphatidylcholine biosynthesis under inhibitory conditions was measured using cells which had been preincubated and labeled with medium containing 50 μmol/L ET-18-OCH3 (●, A) or 50 μmol/L HePC (▲, B). Values are given in dpm/10^6 cells ± SD (n = 3). Abbreviations as in Figure 2.

We also showed that the inhibition of phosphatidylcholine biosynthesis by ET-18-OCH_3 and HePC was reversible (Fig. 4). Six hours after removal of ET-18-OCH_3 and HePC from the medium, incorporation of [*methyl*-^{3}H]choline into phosphatidylcholine continued and exceeded the level of non-treated controls.

It has been accepted that phosphatidylcholine biosynthesis is regulated by translocation of the rate-limiting enzyme (CTP:phosphocholine cytidylyltransferase) from the cytosol to membranes where the enzyme becomes active (20). In order to investigate the effect of ET-18-OCH_3 and HePC on the localization of cytidylyltransferase, we permeabilized cells with digitonin and measured the enzyme activity in the digitonin supernatant and in the remaining cell ghosts. When cells were incubated for 4 h with 50 μmol/L ET-18-OCH_3, the amount of membrane-bound cytidylyltransferase was decreased by 60% as compared with controls (Table 1). Addition of 50 μmol/L HePC to the medium caused a 50% decrease of membrane-bound cytidylyltransferase which is nearly the same as reported previously (17).

We also measured the influence of both synthetic phospholipids on liposome-activated cytidylyltransferase *in vitro*. For this purpose, we prepared liposomes (100 μmol/L oleic acid/100 μmol/L phosphatidylcholine) which contained 50 μmol/L of ET-18-OCH_3 or HePC. Whereas liposome-activated cytidylyltransferase was inhibited by addition of HePC by 38.1 ± 3.7% as compared with controls, cytidylyltransferase activity was not significantly affected by ET-18-OCH_3 in this *in vitro* system. Cytidylyltransferase activity in the presence of ET-18-OCH_3 was 110 ± 18% as compared with controls.

DISCUSSION

Recently, we showed that alkylphosphocholines, such as HePC, inhibit cell proliferation (9) and phosphatidylcholine biosynthesis (9,21). Both cell proliferation and phosphatidylcholine biosynthesis were stimulated by tumor-promoting phorbol esters, such as TPA, while the TPA-induced effects were counteracted by HePC (8,10). We therefore suggested that a link exists between the anti-proliferative effect of HePC and its inhibitory properties on phosphatidylcholine biosynthesis. In the present study, we investigated the effect of the alkyllysophospholipid ET-18-OCH_3 on cell proliferation and phosphatidylcholine biosynthesis and compared its effects with those of the alkylphosphocholine, HePC. This was done to further elucidate the mechanisms that might account for the antineoplastic action of the different synthetic phospholipids. Up to now various mechanisms of action, including the disturbance of phospholipid metabolism (3), effects on membrane cholesterol (22), activation of macrophages (23), and inhibition of cellular enzymes, such as protein kinase C (10, 24,25) and (Na^+-K^+)-activated adenosine triphosphatase (24), have been proposed. It was also reported that ET-18-OCH_3 acts as an activator of protein kinase C in HL-60 cells (26).

TABLE 1
Influence of ET-18-OCH_3 and HePC on the Distribution of Cytidylyltransferase in MDCK Cells[a]

	Control		ET-18-OCH_3		HePC	
	Cytosol (%)	Membrane (%)	Cytosol (%)	Membrane (%)	Cytosol (%)	Membrane (%)
3 min of digitonin	59.4 ± 3.0	40.6 ± 2.5	82.9 ± 12.9	17.1 ± 3.1[b]	79.6 ± 1.0	20.4 ± 0.7[b]

[a]The translocation of cytidylyltransferase in Madin-Darby canine kidney (MDCK) cells was measured by digitonin-mediated release of cytosolic cytidylyltransferase. Cells were incubated for 4 h with medium containing either 50 μmol/L 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3), 50 μmol/L hexadecylphosphocholine (HePC), or no supplements as a control. After incubation, cytidylyltransferase activity was measured in supernatants obtained after 3 min of digitonin treatment and in the cell ghost homogenates as described in the Experimental Procedures section. The distribution of cytosolic and membrane-bound cytidylyltransferase activity was calculated in % for each dish (n = 3). The total activity of control cells was 1.2 ± 0.2 nmol/min per 10^6 cells. The total activity was not influenced by treatment of the cells with ET-18-OCH_3 or HePC.
[b]Different from controls with $P < 0.001$.

Our data show that ET-18-OCH_3 is a potent inhibitor of cell proliferation (IC_{50} 75 μmol/L). The agent also inhibited phosphatidylcholine synthesis in confluent MDCK cells (IC_{50} 40 μmol/L), which is in agreement with what was found in an earlier study using leukemic cells (27). Furthermore, we demonstrated that proliferating cells were more susceptible to inhibition than confluent cells (IC_{50} 20 μmol/L for inhibition of phosphatidylcholine biosynthesis). At concentrations >50 μmol/L, ET-18-OCH_3 also showed nonspecific, cytotoxic effects. For example, there was a strong correlation between cytotoxicity and inhibition of phosphatidylcholine biosynthesis ($r = 0.993$).

The situation was somewhat different when cells were treated with HePC. Concerning cell proliferation (IC_{50} 135 μmol/L) and phosphatidylcholine biosynthesis (IC_{50} 67 μmol/L), the IC_{50}-values of HePC were higher as compared with ET-18-OCH_3. However, the effect of HePC on phosphatidylcholine biosynthesis was more specific as there was only a weak correlation between cytotoxicity and inhibition of phosphatidylcholine biosynthesis ($r = 0.736$). The effect of both agents on phosphatidylcholine biosynthesis was reversible, which confirmed our assumption that this effect is not simply due to cell death.

In cells treated with ET-18-OCH_3 or HePC, the inhibition of choline incorporation into phosphatidylcholine was accompanied by an accumulation of radiolabel in the phosphocholine pool (data not shown), indicating that CTP:phosphocholine cytidylyltransferase is the target of the synthetic phospholipids. In order to investigate the mechanism underlying the inhibition of phosphatidylcholine biosynthesis in more detail, the effect of both synthetic phospholipids on the translocation of CTP:phosphocholine cytidylyltransferase was measured. Both agents inhibited translocation of cytidylyltransferase to membranes and, thereby, its activation. Cytidylyltransferase is known to be affected by the lipid composition of cellular membranes (28,29). In a recent study, evidence has been provided that the binding of this enzyme to membranes is modulated by the ratio of bilayer- to nonbilayer-forming lipids (30). We therefore suggest that both synthetic phospholipids disturb the physical properties of the membrane bilayer and, finally, the binding of cytidylyltransferase to membranes.

In conclusion, both types of antiproliferative agents inhibit phosphatidylcholine biosynthesis by disturbing the translocation of the rate-limiting enzyme of this metabolic pathway, CTP:phosphocholine cytidylyltransferase. Therefore, we suggest a link between the regulation of phosphatidylcholine biosynthesis by cytidylyltransferase and the proliferative properties of cells. In this context, evidence has been provided that cytidylyltransferase is translocated to the nuclear envelope in HeLa cells under stimulatory conditions (31).

ACKNOWLEDGMENTS

This study was supported by two grants of the Deutsche Forschungsgemeinschaft (Ge 641/3-1 and Ge 641/3-2).

REFERENCES

1. Berdel, W.E., Andreesen, R., and Munder, P.G. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J.F., ed.) Vol. 2, pp. 41–77, CRC Press, Boca Raton.
2. Kosano, H., and Takatani, O. (1988) *Cancer Res. 48,* 6033–6036.
3. Modolell, M., Andreesen, R., Pahlke, W., Brugger, U., and Munder, P.G. (1979) *Cancer Res. 39,* 4681–4686.
4. Herrmann, D.B.J., and Neumann, M.A. (1986) *J. Biol. Chem. 261,* 7742–7747.
5. Lu, X., and Arthur, G. (1992) *Cancer Res. 52,* 2806–2812.
6. Lu, X., and Arthur, G. (1992) *Cancer Res. 52,* 2813–2817.
7. Unger, C., Eibl, H., Breiser, A., von Heyden, H.W., Engel, J., Hilgard, P., Sindermann, H., Peukert, M., and Nagel, G.A. (1988) *Onkologie 11,* 295–296.
8. Wieder, T., Geilen, C.C., and Reutter, W. (1993) *Biochem. J. 291,* 561–567.
9. Geilen, C.C., Haase, A., Wieder, T., Arndt, D., Zeisig, R., and Reutter, W. (1994) *J. Lipid Res. 35,* 625–632.
10. Geilen, C.C., Haase, R., Buchner, K., Wieder, T., Hucho, F., and Reutter, W. (1991) *Eur. J. Cancer 27,* 1650–1653.
11. Geilen, C.C., Samson, A., Wieder, T., Wild, H., and Reutter, W. (1992) *J. Labelled Comp. Radiopharm. 31,* 1071–1076.
12. Culvenor, J.G., Harris, A.W., Mandel, T.E., Whitelaw, A., and Ferber, E. (1981) *J. Immunol. 126,* 1974–1977.
13. Gillies, R.J., Didier, N., and Denton, M. (1986) *Anal. Biochem. 159,* 109–113.
14. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911–917.
15. Touchstone, J.C., Chen, J.C., and Beaver, K.M. (1980) *Lipids 15,* 61–62.
16. Pelech, S.L., Cook, H.W., Paddon, H.B., and Vance, D.E. (1984) *Biochim. Biophys. Acta 795,* 433–440.
17. Geilen, C.C., Wieder, T., and Reutter, W. (1992) *J. Biol. Chem. 267,* 6719–6724.
18. Sohal, P.S., and Cornell, R.B. (1990) *J. Biol. Chem. 265,* 11746–11750.
19. Smith, P.K., Krohn, R.I., Hermanson, G.T., Malla, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) *Anal. Biochem. 150,* 76–85.
20. Vance, D.E. (1989) in *Phosphatidylcholine Metabolism* (Vance, D.E., ed.) pp. 33–45, CRC Press, Boca Raton.
21. Haase, R., Wieder, T., Geilen, C.C., and Reutter, W. (1991) *FEBS Lett. 288,* 129–132.
22. Diomede, L., Piovani, B., Modest, E.J., Noseda, A., and Salmona, M. (1991) *Int. J. Cancer 49,* 409–413.
23. Yamamoto, N., and Ngwenya, B.Z. (1987) *Cancer Res. 47,* 2008–2013.
24. Zheng, B., Oishi, K., Shoji, M., Eibl, H., Berdel, W.E., Hajdu, J., Vogler, W.R., and Kuo, J.F. (1990) *Cancer Res. 50,* 3025–3031.
25. Überall, F., Oberhuber, H., Maly, K., Zaknun, J., Demuth, L., and Grunicke, H.H. (1991) *Cancer Res. 51,* 807–812.
26. Heesbeen, E.C., Verdonck, C.F., Hermans, S.W.G., van Heugten, H.G., Staal, G.E.J., and Rijksen, G. (1991) *FEBS Lett. 290,* 231–234.
27. Vogler, W.R., Whigham, E., Bennett, W.D., and Olson, A.C. (1985) *Exp. Hematol. 13,* 629–633.
28. Cornell, R.B. (1991) *Biochemistry 30,* 5873–5880.
29. Cornell, R.B. (1991) *Biochemistry 30,* 5881–5888.
30. Jamil, H., Hatch, G.M., and Vance, D.E. (1993) *Biochem. J. 291,* 419–427.
31. Wang, Y., MacDonald, J.I.S., and Kent, C. (1993) *J. Biol. Chem. 268,* 5512–5518.

[Received May 5, 1994, and in revised form February 13, 1995; Revision accepted February 23, 1995]

Iron-Catalyzed Reaction Products of α-Tocopherol with Methyl 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoate

Ryo Yamauchi*, Norio Yamamoto and Koji Kato

Department of Food Science, Faculty of Agriculture, Gifu University, Gifu 501-11 Japan

ABSTRACT: α-Tocopherol was reacted with methyl 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate in the presence of an iron-chelate, Fe(III)-acetylacetonate, at 37°C in benzene. The reaction was carried out either aerobically or anaerobically. The main products of α-tocopherol under air were isolated and identified as two stereoisomers of 4a,5-epoxy-8a-hydroperoxy-α-tocopherone, four stereoisomers of methyl 9-(8a-dioxy-α-tocopherone)-12,13-epoxy-10(*E*)-octadecenoate, four stereoisomers of methyl 11-(8a-dioxy-α-tocopherone)-12,13-epoxy-9(*Z*)-octadecenoate, two stereoisomers of methyl 13(*S*)-(8a-dioxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoate, and α-tocopherol dimer. Besides the 8a-(lipid-peroxy)-α-tocopherones, two stereoisomers of methyl 11-(α-tocopheroxy)-12(*S*),13(*S*)-epoxy-9(*E*)-octadecenoate, two stereoisomers of methyl 9-(α-tocopheroxy)-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoate, and two isomers of methyl (α-tocopheroxy)-octadecadienoate were obtained under nitrogen atmosphere. The results indicate that the peroxyl radicals from lipid hydroperoxides prefer to react with the 8a-carbon radical of α-tocopherol and the carbon-centered radicals react with the phenoxyl radical of α-tocopherol.

Lipids 30, 395–404 (1995).

The primary products of lipid peroxidation are hydroperoxides, which can dissociate into free radicals. Lipid hydroperoxides are fairly stable at physiological temperatures, and a major role of transition metals is to catalyze their decomposition (1,2). Transition metal ions catalyze homolysis of lipid hydroperoxides that are cleaved to alkylperoxyl radicals by metal ions in the oxidized state, such as Fe(III), whereas reduced metal ions, such as Fe(II), lead to alkoxyl radicals. The free radicals produced in these processes are believed to stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms. α-Tocopherol (TOH), an antioxidant in foods and living cells, can trap such free radicals to stop lipid peroxidation and converts them to nonradical products (3).

Free radical reactions of TOH take place *via* the α-tocopheroxyl radical as intermediate (4,5). If a suitable free radical is present, a nonradical product may be formed from the coupling of the free radical with α-tocopheroxyl radical. The reaction products of TOH with lipid-peroxyl radicals have been reported to be 8a-(lipid-peroxy)-α-tocopherones (6–10). TOH also traps alkyl and alkoxyl radicals to give alkylated products (11–13). In our previous study (11), we characterized isomeric 6-*O*-(lipid-alkyl)-α-tocopherols as the reaction products of TOH with the alkyl radicals of methyl linoleate. It has been reported that the iron-catalyzed decomposition of lipid hydroperoxides under anaerobic conditions yielded epoxyalkyl radicals which, in turn, reacted with α-tocopheroxyl radical to form 6-*O*-epoxyalkyl-α-tocopherols (12,13). However, the reaction products of TOH during metal-catalyzed decomposition of lipid hydroperoxides, especially under aerobic conditions, are still unknown.

In this study, we have isolated and characterized the reaction products of TOH with free radicals formed by iron-catalyzed decomposition of methyl 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate (13-MLOOH) under air or nitrogen atmosphere. A reaction mechanism of TOH with free radicals from 13-MLOOH is discussed based on the reaction products observed.

MATERIALS AND METHODS

Materials. RRR-TOH was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by reversed-phase high-performance liquid chromatography (HPLC) using methanol. 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoate (13-LOOH) was prepared by oxidation of linoleic acid (99% purity; Wako Pure Chemical Industries, Osaka, Japan) with soybean lipoxygenase-1 (Sigma) (14). The 13-LOOH was converted to its methyl ester with diazomethane in diethyl ether and purified by silica-gel column chromatography using a mixture

*To whom correspondence should be addressed at Department of Food Science, Faculty of Agriculture, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-11, Japan.

Abbreviations: CD, circular dichroism; Fe(III)AA, Fe(III) acetylacetonate; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; IR, infrared; 13-LOOH, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13-MLOOH, methyl 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate; MS, mass spectra; NMR, nuclear magnetic resonance; TOH, α-tocopherol; UV, ultraviolet.

Copyright © 1995 by AOCS Press

of hexane/diethyl ether. The purity of 13-MLOOH was checked by normal-phase HPLC after reduction (15). The purity of 13-MLOOH was about 90% of the total MLOOH, the remainder being the 9-isomer. Fe(III) acetylacetonate [Fe(III)AA] was purchased from Dojindo Laboratories (Kumamoto, Japan). All solvents were distilled in an all-glass still before use.

Apparatus. HPLC was performed using a Jasco Trirotar pump and a Model GP-A40 gradient programmer (Japan Spectroscopic Co., Tokyo, Japan). A Jasco Model 875-ultraviolet (UV) detector or a Model 820-FP spectrofluorometer was used as the detector. ^{1}H (270.17 MHz) and ^{13}C (67.9 MHz) nuclear magnetic resonance (NMR) spectra were recorded at 25°C on a JEOL (Tokyo, Japan) JNM-GX-270 FT NMR spectrometer using $CDCl_3$ as solvent and tetramethylsilane as internal standard. Mass spectra (MS) were obtained on a GCMS QP-1000 instrument (Shimadzu Co., Kyoto, Japan) operated in the electron impact mode (70 eV). Samples were introduced *via* the direct inlet probe. Gas chromatography/mass spectrometry (GC/MS) was done on the same instrument equipped with a Shimadzu Hicap CBP-1 capillary column (25 m × 0.2 mm), and the temperature was programmed from 230 to 290°C (4°C/min). Helium was used as carrier gas at a flow rate of 0.8 mL/min. UV spectra were measured with a Jasco Ubest-30 spectrophotometer. Infrared (IR) spectra of samples in liquid film were measured on a Jasco A-302 IR spectrometer. Specific rotations were determined with a Union PM-201 automatic digital polarimeter (Union Gikken, Osaka, Japan). Circular dichroism (CD) measurements of the optical isomers of the compounds were performed with a Jasco J-600 spectropolarimeter.

Reaction procedure. 13-MLOOH (2.0 g, 6.1 mmol) and TOH (0.53 g, 1.2 mmol) were dissolved in 600 mL of benzene. Fe(III)AA (0.43 g, 1.2 mmol) was then added to the reaction mixture and incubated at 37°C for 20 h under air. When the reaction was carried out under anaerobic conditions, nitrogen was bubbled through the reaction mixture. After the reaction was complete, the solvent was removed *in vacuo*. The oily residue was dissolved in hexane (10 mL), applied onto a silica gel column (20 g). The products were separated from the Fe(III)AA catalyst by rapid elution with 100 mL of hexane/diethyl ether (1:1, vol/vol) by applying reduced pressure to the column outlet. The solvent of the eluent was removed *in vacuo*, and oily products were obtained. The products were dissolved in ethanol and analyzed by reversed-phase HPLC. Reversed-phase HPLC was done with a μBondasphere 5μC_{18} column (3.9 × 150 mm; Nihon Waters Co., Tokyo, Japan) developed with methanol/ethanol (3:2, vol/vol) at a flow rate of 1.0 mL/min. The eluent was monitored by an absorbance at 240 nm. For isolation of the reaction products, a WakoSil 5C18 column (1.0 × 300 cm; Wako) was developed with the same solvent at 5.0 mL/min. The dimer in the column was eluted using ethanol as the solvent. Normal-phase HPLC was done with a Bio-Sil D 90-10 column (1.0 × 250 mm; Bio-Rad Laboratories, Tokyo, Japan) developed with hexane/2-propanol (100:0.3 or 100:0.5, vol/vol) at a flow rate of 5.0 mL/min. The compounds isolated were dissolved in ethanol and stored at −20°C until analysis.

A mixture of 13-MLOOH (10 mM), TOH (2 mM), and Fe(III)AA (2 mM) in benzene was incubated at 37°C under air. At regular intervals, aliquots of the sample solution were withdrawn, passed through a Sep-Pak silica cartridge (Waters, Millford, MA) using 3 mL of hexane/diethyl ether (1:1, vol/vol), and the eluent was dried, *in vacuo*. The residue was dissolved in ethanol and injected into the HPLC. A μBondasphere 5μC_8 column (3.9 × 150 mm) was developed with a 15-min linear gradient of acetonitrile/methanol/water (60:35:5, by vol) to acetonitrile/2-propanol/hexane (35:50:15, by vol) at a flow rate of 1.0 mL/min (11). The amounts of TOH were calculated from the peak area by monitoring the fluorescence (ex. 295 nm, em. 340 nm). The amounts of reaction products were calculated from the peak areas by monitoring elutions at 240 nm. α-Tocopheryl acetate was used as the internal standard.

Derivatization. The compounds isolated (*ca.* 2 mg) were dissolved in 1 mL of methanol/ethanol (2:1, vol/vol) and reduced with sodium borohydride at 4°C for 2 h, extracted with diethyl ether, converted to the corresponding trimethylsilyl ethers, and then analyzed by GC/MS. Furthermore, the methyl epoxyhydroxyoctadecenoates in the reduced compounds were separated by normal-phase HPLC using a Bio-Sil D 90-10 column (1 × 25 cm) and hexane/2-propanol (100:4, vol/vol) at 5 mL/min. The eluent was monitored by absorbance at 203 nm. The *R* and *S* configurations of the hydroxy group were determined by CD spectroscopy in methanol (8).

RESULTS

Reaction products under aerobic conditions. Transition metal ions are effective in catalyzing free radical reactions in the presence of hydroperoxides and TOH (12,13). Figure 1 shows the Fe(III)AA-catalyzed reaction products of TOH with 13-MLOOH under air. Six peaks that appeared to be the reaction products of TOH were collected by preparative reversed-phase HPLC. The dimer fraction was eluted from the column with ethanol, obtained as a yellow oil (**5**, 50.7 mg yield), and identified by the HPLC behavior with that of the authentic sample as the spirodiene dimer of TOH (16). Each peak was further separated by normal-phase HPLC using hexane/2-propanol (100:0.5, vol/vol) as eluate. Thus, compounds **1a**, **1b**, **2a**, **2b**, **2c**, **3a**, **3c**, **3d**, **4a**, and **4b** were obtained, and **2d** and **3b** were obtained as a mixture. The structures of compounds **1–4** were determined as described below (Scheme 1).

Compounds **1a** and **1b** were obtained as colorless oils (yields: **1a**, 15.8 mg; **1b**, 13.3 mg). Their structures were shown to be a pair of diastereoisomers of 4a,5-epoxy-8a-hydroperoxy-α-tocopherone as reported by Matsuo *et al.* (7). **1a**: UV (ethanol) λ_{max} 248 nm (ε 6000); IR (film) ν_{max} 3360, 2930, 1680, 1460, 1380, 1260, 1230, 1140, 1090, 1060, 950 cm^{-1}; MS *m/z* 445 ($[M - HO_2]^+$, 4%), 430 (1), 428 (1), 419

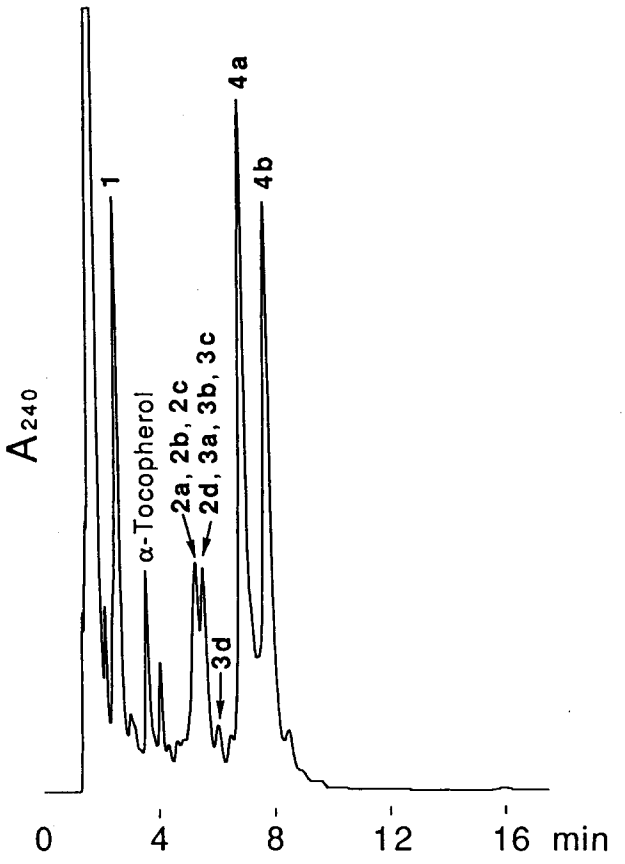
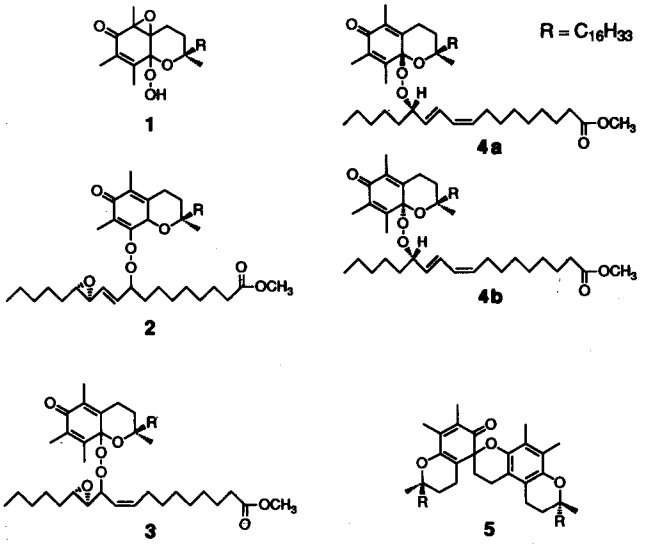


FIG. 1. Reversed-phase high-performance liquid chromatography (HPLC) of the products of the Fe(III) acetylacetone-catalyzed reaction of methyl 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate and α-tocopherol under air for 20 h. HPLC was done with a μBondasphere 5μC_{18} (Nihon Waters Co., Tokyo, Japan) column (3.9 × 150 mm) which was developed with methanol/ethanol (3:2, vol/vol) at 1.0 mL/min.



SCHEME 1

(2), 417 (2), 416 (3), 205 (3), 165 (8), 43 (100). ^{1}H NMR ($CDCl_3$) δ 0.84–0.88 (*m*, 12H), 1.09–1.96 (*m*, 23H), 1.32 (*s*, 3H), 1.48 (*s*, 3H), 1.82 and 1.83 (*s*, 3H), 1.89 and 1.90 (*s*, 3H), 2.09 (*m*, 1H), 2.52 (*m*, 1H), 7.49 (*s*, 1H); $[\alpha]_D^{25}$ + 81 (c 0.41, ethanol). **1b**: UV (ethanol) λ_{max} 247 (ε 5900), IR (film) ν_{max} 3360, 2930, 1680, 1460, 1380, 1260, 1230, 1140, 1090, 1060, 950 cm^{-1}; MS *m/z* 445 ($[M - HO_2]^+$, 4%), 430 (1), 428 (3), 419 (3), 417 (2), 416 (3), 205 (3), 165 (6), 43 (100); ^{1}H NMR ($CDCl_3$) δ 0.83–0.88 (*m*, 12H), 1.08–1.90 (*m*, 23H), 1.46 (*s*, 3H), 1.49 (*s*, 3H) 1.82 and 1.83 (*s*, 3H), 1.90 (*s*, 3H), 2.01 (*m*, 1H), 2.56 (*m*, 1H), 7.54 (*s*, 1H); $[\alpha]_D^{25}$ – 82 (c 0.28, ethanol). It is obvious that the configurations of the epoxy and hydroperoxy groups in **1** are responsible for the structural difference between **1a** and **1b**. However, the stereochemistry of **1a** and **1b** remains to be assigned.

Compounds **2** and **3** were obtained as colorless oils (yields: **2a**, 15.8 mg; **2b**, 13.3 mg; **2c**, 15.3 mg; **3a**, 5.9 mg; a mixture of **2d** and **3b**, 15.0 mg; **3c**, 7.2 mg; **3d**, 6.3 mg). Each compound gave a positive peroxide test (17). UV spectra of these compounds showed the same absorption maximum at 241 nm in ethanol (**2a**, ε 11500; **2b**, ε 12900; **2c**, ε 13000; **3a**, ε 10800; a mixture of **2d** and **3b**, ε 12200; **3c**, ε 12200; **3d**, 11700), indicating the presence of a dienone structure (6).

Compounds **2** and **3** were reduced with sodium borohydride, and the products were identified by GC/MS as trimethylsilyl derivatives. Compounds **2a**, **2b**, and **2c** gave two major peaks that were identified to be methyl 12,13-epoxy-9-hydroxy-10-octadecenoate trimethylsilyl ether (18): MS *m/z* 398 (M^+, 1%), 383 ($[M - 15]^+$, 2), 327 (3), 259 (α-cleavage of the 9-trimethylsilyloxy group, 16), 241 (α-cleavage of the 9-trimethylsilyloxy group, 70), 103 (23), 99 (100), 73 (85); and TOH trimethylsilyl ether (9): MS *m/z* 502 (M^+, 100%), 237 (56%), 73 (79%). Compounds **3a**, **3c**, and **3d** gave methyl 12,13-epoxy-11-hydroxy-9-octadecenoate trimethylsilyl ether (18): MS *m/z* 383 ($[M - 15]^+$, 1%), 327 (2), 285 (α-cleavage of the 11-trimethylsilyloxy group, 80), 163 (6), 155 (12), 135 (16), 129 (21), 73 (100); and TOH trimethylsilyl ether. A mixture of compounds **2d** and **3b** gave trimethylsilyl ethers of methyl 12,13-epoxy-9-hydroxy-10-octadecenoate, methyl 12,13-epoxy-11-hydroxy-9-octadecenoate and TOH. The results indicate that compounds **2a**, **2b**, **2c**, and **2d** consist of methyl 12,13-epoxy-9-hydroxy-10-octadecenoate moiety and TOH moiety in the molecule; and **3a**, **3b**, **3c**, and **4**, methyl 12,13-epoxy-11-hydroxy-9-octadecenoate moiety and TOH moiety, respectively.

The configurations of the hydroxy group of methyl 12,13-epoxy-9-hydroxy-10-octadecenoates from compounds **2a–2d** were determined by CD measurement. Methyl 12,13-epoxy-9-hydroxy-10-octadecenoate from compounds **2c** and **2d** showed a positive Cotton effect and those from compounds **2a** and **2b** showed a negative Cotton effect, respectively. Therefore, the hydroxy group of methyl 12,13-epoxy-9-hydroxy-10-octadecenoate from compounds **2c** and **2d** is assumed to be in the *S* configuration, and that from **2a** and **2b** is in the *R* configuration (9). The methyl 12,13-epoxy-11-hydroxy-9-octadecenoates from compounds **3a**, **3b**, **3c** and **3d** did not show a clear Cotton effect.

TABLE 1
^{1}H Nuclear Magnetic Resonance Chemical Shifts of Methyl 9-(8a-dioxy-α-tocopherone)-epoxy-10(*E*)-octadecenoates[a]

2a	2b	2c	2d	Assignment[b]
0.80–0.92 (*m*, 15H)	0.84–0.92 (*m*, 15H)	0.80–0.93 (*m*, 15H)	0.80–0.90 (*m*, 15H)	H_l-18, H_t-4'a, H_t-8'a, H_t-12'a, H_t-13'
1.05–2.10 (*m*, 43H)	1.12–2.25 (*m*, 43H)	1.04–2.25 (*m*, 43H)	1.04–2.05 (*m*, 43H)	H_l-3 to H_l-8, H_l-14 to H_l-17, H_t-3, H_t-1' to H_t-12'
1.32 (*s*, 3H)	1.06 (*s*, 3H)	1.07 (*s*, 3H)	1.31 (*s*, 3H)	H_t-2a
1.86 (*s*, 3H)	1.85 (*s*, 3H)	1.86 (*s*, 3H)	1.85 (*s*, 3H)	H_t-7a
1.88 (*s*, 3H)	1.87 (*s*, 3H)	1.87 (*s*, 3H)	1.85 (*s*, 3H)	H_t-5a
1.94 (*s*, 3H)	1.93 (*s*, 3H)	1.93 (*s*, 3H)	1.93 (*s*, 3H)	H_t-8b
2.30 (*t*,*J* = 7.5 Hz, 2H)	2.29 (*t*,*J* = 7.6 Hz, 2H)	2.30 (*t*,*J* = 7.5 Hz, 2H)	2.30 (*t*,*J* = 7.5 Hz, 2H)	H_l-2
2.60 (*t*,*J* = 6.4 Hz, 2H)	2.60 (*t*,*J* = 6.6 Hz, 2H)	2.60 (*br*,*t*, 2H)	2.59 (*t*,*J* = 6.6 Hz, 2H)	H_t-4
2.79 (*dt*,*J* = 2.2, 5.2 Hz, 1H)	2.79 (*dt*,*J* = 1.9, 5.3 Hz, 1H)	2.79 (*dt*,*J* = 2.0, 5.3 Hz, 1H)	2.80 (*dt*,*J* = 2.0, 5.4 Hz, 1H)	H_l-13
3.08 (*dd*,*J* = 2.0, 7.8 Hz, 1H)	3.06 (*dd*,*J* = 1.9, 7.7 Hz, 1H)	3.07 (*dd*,*J* = 1.9, 7.9 Hz, 1H)	3.07 (*dd*,*J* = 1.9, 7.9 Hz, 1H)	H_l-12
3.66 (*s*, 3H)	3.66 (*s*, 3H)	3.66 (*s*, 3H)	3.66 (*s*, 3H)	CH_3O
4.12 (*q*,*J* = 6.8 Hz, 1H)	4.15 (*q*,*J* = 6.6 Hz, 1H)	4.12 (*q*,*J* = 6.8 Hz, 1H)	4.14 (*q*,*J* = 6.8 Hz, 1H)	H_l-9
5.31 (*dd*,*J* = 7.6, 15.9 Hz, 1H)	5.34 (*dd*,*J* = 7.8, 15.7 Hz, 1H)	5.29 (*dd*,*J* = 7.8, 15.5 Hz, 1H)	5.34 (*dd*,*J* = 7.9, 15.6 Hz, 1H)	H_l-11
5.79 (*dd*,*J* = 7.4, 15.7 Hz, 1H)	5.74 (*dd*,*J* = 7.1, 15.6 Hz, 1H)	5.74 (*dd*,*J* = 7.6, 15.5 Hz, 1H)	5.74 (*dd*,*J* = 7.2, 15.6 Hz, 1H)	H_l-10

[a]Shifts in parts per million downfield relative to tetramethylsilane. Multiplicity: *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; *br*, broad.

[b]The numbering systems used are the standard numbering systems for the fatty acid chains and the α-tocopoherol moiety: H_l, the hydrogen atom in the fatty acid moiety; H_t, the hydrogen atom in the α-tocopherol moiety.

The ^{1}H NMR spectra of the compounds were consistent with those expected for methyl 9-(8a-dioxy-α-tocopherone)-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoates and methyl 11-(8a-dioxy-α-tocopherone)-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoates (Tables 1 and 2). In particular, the geometrical relationship between the compounds can be established by interpretation of the spectra. Since the 8a-peroxy group of the tocopherone moiety to the 2-methyl group of the tocopherone is *trans* or *cis*, shielding (**2b**, δ 1.06; **2c**, δ 1.07; **3b**, δ 1.08; **3c**, δ 1.07) or deshielding (**2a**, δ 1.32; **2d**, δ 1.31; **3a**, δ 1.38; **3d**, δ 1.34) of the 2-methyl group is observed (9). On the *erythro–threo* isomerism of *vic*-epoxyhydroxy compounds, the

TABLE 2
^{1}H Nuclear Magnetic Resonance Chemical Shifts of Methyl 11-(8a-Dioxy-α-tocopherone)-epoxy-9(*Z*)-octadecenoates[a]

3a	3b	3c	3d	Assignment[b]
0.80–0.90 (*m*, 15H)	0.88–0.90 (*m*, 15H)	0.80–0.88 (*m*, 15H)	0.81–0.90 (*m*, 15H)	H_l-18,H_t-4'a, H_t-8'a, H_t-12'a, H_t-13'
1.05–2.15 (*m*, 43H)	1.05–2.05 (*m*, 43H)	1.05–2.05 (*m*, 43H)	1.06–2.10 (*m*, 43H)	H_l-3 to H_l-8, H_l-14 to H_l-17, H_t-3, H_t-1' to H_t-12'
1.38 (*s*, 3H)	1.08 (*s*, 3H)	1.07 (*s*,3H)	1.34 (*s*, 3H)	H_t-2a
1.85 (*s*, 3H)	1.87 (*s*, 3H)	1.85 (*s*, 3H)	1.85 (*s*, 3H)	H_t-7a
1.87 (*s*, 3H)	1.87 (*s*, 3H)	1.86 (*s*, 3H)	1.87 (*s*, 3H)	H_t-5a
1.97 (*s*, 3H)	1.94 (*s*, 3H)	1.98 (*s*, 3H)	1.96 (*s*, 3H)	H_t-8b
2.30 (*t*,*J* = 7.6 Hz, 2H)	2.29 (*t*,*J* = 7.5 Hz, 2H)	2.30 (*t*,*J* = 7.5 Hz, 2H)	2.30 (*t*,*J* = 7.5 Hz, 2H)	H_l-2
2.61 (*t*,*J* = 6.6 Hz, 2H)	2.59 (*t*,*J* = 6.6 Hz, 2H)	2.62 (*br*,*t*, 2H)	2.61 (*t*,*J* = 6.7 Hz, 2H)	H_t-4
2.70 (*m*, 1H)	2.70 (*dt*,*J* = 1.9, 5.6 Hz, 1H)	2.67 (*m*, 1H)	2.75 (*dt*,*J* = 2.2, 5.4 Hz, 1H)	H_l-13
2.81 (*dd*,*J* = 2.2, 6.1 Hz, 1H)	2.72 (*dd*,*J* = 1.9, 5.0 Hz, 1H)	2.77 (*dd*,*J* = 2.4, 6.0 Hz, 1H)	2.79 (*dd*,*J* = 2.3, 5.3, Hz, 1H)	H_l-12
3.67 (*s*, 3H)	3.66 (*s*, 3H)	3.67 (*s*, 3H)	3.67 (*s*, 3H)	CH_3O
4.30 (*dd*,*J* = 6.1, 9.2 Hz, 1H)	4.36 (*dd*,*J* = 5.3, 8.6 Hz, 1H)	4.35 (*dd*,*J* = 5.8, 9.2 Hz, 1H)	4.35 (*dd*,*J* = 5.2, 8.6 Hz, 1H)	H_l-11
5.20 (*dd*,*J* = 9.1, 10.8 Hz, 1H)	5.23 (*dd*,*J* = 8.9, 10.8 Hz. 1H)	5.20 (*dd*,*J* = 9.2, 10.9 Hz, 1H)	5.20 (*dd*,*J* = 8.8, 10.8 Hz, 1H)	H_l-10
5.60 (*dt*,*J* = 7.6, 10.8 Hz, 1H)	5.64 (*dt*,*J* = 7.8, 10.8 Hz, 1H)	5.59 (*dt*,*J* = 7.5, 10.6 Hz, 1H)	5.64 (*dt*,*J* = 7.5, 10.9 Hz, 1H)	H_l-9

[a]Shifts in parts per million downfield relative to tetramethylsilane. Multiplicity: *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; *br*, broad.

[b]The numbering systems used are the standard numbering systems for the fatty acid chains and the α-tocopoherol moiety: H_l, the hydrogen atom in the fatty acid moiety; H_t, the hydrogen atom in the α-tocopherol moiety.

TABLE 3
^{13}C Nuclear Magnetic Resonance Chemical Shifts of Methyl 9-(8a-dioxy-α-tocopherone)-epoxy-10(*E*)-octadecenoates[a]

2a	2b	2c	2d	Assignment[b]
10.8	10.7	10.8	10.7	C_t-5a
11.2	11.3	11.2	11.3	C_t-7a
13.5	13.6	13.5	13.7	C_t-8b
14.0	14.0	14.0	14.0	C_l-18
19.7, 19.8	19.7, 19.8	19.7, 19.8	19.7, 19.8	C_t-4'a, C_t-8'a
21.3	21.1	21.1	21.4	C_t-2'
22.3	22.2	22.5	22.0	C_t-4
22.6 (2)[c], 22.7	22.6 (2), 22.7	22.6 (2), 22.7	22.6, 22.7, 22.8	C_l-17, C_t-12'a, C_t-13'
24.4, 24.8	24.5, 24.8	24.5, 24.8	24.4, 24.8	C_t-6, C_t-10'
24.9, 25.5, 26.8	24.9, 25.4, 25.6	24.9, 25.3, 25.6	24.9, 25.6, 26.9	C_l-3, C_l-15, C_t-2a
28.0 (2), 29.0 (3), 29.3, 31.6, 31.9, 33.5	28.0 (2), 29.0 (3), 29.3, 31.6, 32.0, 33.0	28.0 (2), 29.0 (3), 29.3, 31.6, 31.9, 33.0	28.0 (2), 29.0 (3), 29.3, 31.6, 32.0, 33.6	C_l-4 to C_l-8, C_l-14, C_l-16, C_t-3, C_t-12'
32.6, 32.8	32.8, 32.9	32.8, 32.9	32.8, 32.9	C_t-4',C_t-8'
34.1	34.1	34.1	34.1	C_l-2
37.3, 37.4 (2), 37.5	37.4, 37.5 (2), 37.6	37.3, 37.5, 37.6 (2)	37.3, 37.5 (2), 37.6	C_t-3', C_t-5', C_t-7', C_t-9'
39.4	39.4	39.4	39.4	C_t-11'
42.1	43.3	43.2	42.1	C_t-1'
51.4	51.4	51.4	51.4	CH_3O
57.9, 60.5	57.9, 60.5	57.8, 60.6	58.0, 60.6	C_l-12, C_l-13
76.8	77.0	76.8	76.9	C_t-2
84.2	83.6	84.2	83.8	C_l-9
97.5	97.1	97.2	97.2	C_t-8a
130.2, 134.9	130.0, 134.8	130.8, 134.8	130.7, 134.8	C_l-10, C_l-11
131.2, 131.4	131.4 (2)	131.4, 131.7	131.0, 131.6	C_t-5, C_t-7
145.6	145.8	145.2	146.0	C_t-4a
148.7	148.6	148.7	148.5	C_t-8
174.3	174.3	174.3	174.3	C_l-1
185.5	185.4	185.4	185.5	C_t-6

[a]Shifts in parts per million downfield relative to tetramethylsilane.

[b]The numbering systems used are the standard numbering systems for the fatty acid chains and the α-tocopoherol moiety: C_l, the carbon atom in the fatty acid moiety; C_t, the carbon atom in the α-tocopherol moiety.

[c]Number of carbon in parentheses.

coupling constants of the *erythro* isomers are smaller than those of the *threo* isomers (13,19). From a consideration of the coupling constants between the protons at C-11 and C-12 of the fatty acid moiety in **3a** (J = 6.1 Hz), **3b** (J = 5.3 Hz), **3c** (J = 5.8 Hz) and **3d** (J = 5.2 Hz), **3a**, **3b**, **3c**, and **3d** appear to be the *threo, erythro, threo* and *erythro* isomers, respectively (Table 2). The magnitude of the olefinic coupling constants (J = 15.5–15.7 Hz in Table 1 and J = 10.6–10.9 Hz in Table 2) was typical of *trans* and *cis* olefins, respectively (20). The coupling constant between the protons at C-12 and C-13 of the fatty acid moiety (J = 1.9–2.4 Hz) was a characteristic of *trans* epoxides (13). The ^{13}C NMR spectra of the compounds show two carbonyls (δ 174.3 and 185.4–185.5), a carbon atom bearing two oxy substituents (δ 97.1–97.6), a secondary ether (δ 80.0–80.7 or 83.6–84.2), a tertiary carbon (δ 76.4–77.0) two epoxy carbons (δ 55.2–58.0 and 58.2–60.6), and a methoxy carbon (δ 51.4–51.5) (Tables 3 and 4).

From these results and other spectral data, the structures of compounds **2** and **3** were identified as stereoisomers of methyl 9-(8a-dioxy-α-tocopherone)-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoate and methyl 11-(8a-dioxy-α-tocopherone)-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoate, respectively. The stereochemistry of each compound was assumed as follows: **2a**, methyl 9(*R*)-[8a(*R*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoate; IR (film) ν_{max} 2930, 1740, 1640, 1460, 1380, 1085, 965, 920 cm^{-1}; MS *m/z* 445 (3%), 430 (7), 417 (3), 219 (2), 205 (3), 203 (7), 165 (12), 137 (8), 43 (100); $[\alpha]_D^{25}$ + 13 (c 0.53, ethanol). **2b**, methyl 9(*R*)-[8a(*S*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoate: IR (film) ν_{max} 2930, 1740, 1640, 1460, 1380, 1080, 965, 920 cm^{-1}; MS *m/z* 445 (3%), 430 (11), 417 (2), 219 (2), 205 (3), 203 (7), 165 (15), 137 (8), 43 (100); $[\alpha]_D^{25}$ +28 (c 0.34, ethanol). **2c**, methyl 9(*S*)-[8a(*S*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoate: IR (film) ν_{max} 2930, 1740, 1640, 1460, 1380, 1085, 965, 920 cm^{-1}; MS *m/z* 445 (2%), 430 (9), 417 (2), 219 (2), 205 (4), 203 (7), 165 (14), 137 (10), 43 (100); $[\alpha]_D^{25}$ – 13 (c 0.36, ethanol). **3a**, methyl 11(*S*)-[8a(*R*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoate: IR (film)

TABLE 4
^{13}C Nuclear Magnetic Resonance Chemical Shifts of Methyl 11-(8a-dioxy-α-tocopherone)-epoxy-9(*Z*)-octadecenoates[a]

3a	3b	3c	3d	Assignment[b]
10.9	10.8	10.9	10.8	C_t-5a
11.3	11.3	11.3	11.4	C_t-7a
13.6	13.7	13.6	13.8	C_t-8b
14.0	14.0	14.0	14.0	C_l-18
19.6, 19.8	19.6, 19.8	19.6, 19.8	19.6, 19.8	C_t-4'a, C_t-8'a
21.4	21.2	21.4	21.4	C_t-2'
22.5	22.3	22.5	22.1	C_t-4
22.7 (2)[c], 22.8	22.7 (2), 22.8	22.7 (2), 22.8	22.6, 22.7 (2)	C_l-17, C_t-12'a, C_t-13'
24.4, 24.8	24.5, 24.8	24.4, 24.8	24.4, 24.8	C_t-6', C_t-10'
25.0, 25.6, 26.8	25.0, 25.3, 25.4	25.0, 25.6, 26.8	25.0, 26.6, 26.9	C_l-3, C_l-15, C_t-2a
28.0, 28.1, 29.1 (2), 29.2 (2), 29.4, 31.5, 31.7	28.1, 29.1, 29.2 (2), 29.3 (2), 31.7, 31.9, 33.6	28.0, 28.1, 29.1 (2), 29.2 (2), 29.4, 31.5, 31.7	28.0, 28.1, 29.1 (2), 29.2, 29.4, 31.6, 31.7, 33.5	C_l-4 to C_l-8, C_l-14, C_l-16, C_t-3, C_t-12'
32.6, 32.8	32.8, 32.9	32.6, 32.8	32.6, 32.8	C_t-4', C_t-8'
34.1	34.1	34.1	34.1	C_l-2
37.3, 37.4 (2), 37.5	37.3, 37.4, 37.5, 37.6	37.3, 37.4 (2), 37.5	37.3, 37.4 37.5 (2)	C_t-3', C_t-5', C_t-7', C_t-9'
39.4	39.4	39.4	39.4	C_t-11'
42.1	43.4	42.1	42.0	C_t-1'
51.5	51.4	51.5	51.4	CH_3O
55.2, 59.2	57.5, 60.6	55.2, 59.2	57.6, 58.2	C_l-12, C_l-13
76.4	76.9	76.4	77.0	C_t-2
80.7	80.0	80.7	79.9	C_l-11
97.5	97.2	97.5	97.6	C_t-8a
122.7	123.6	122.7	123.0	C_l-10
131.1, 131.5	131.4, 131.8	131.1, 131.5	131.3, 131.7	C_t-5, C_t-7
136.7	136.9	136.7	137.4	C_l-9
145.8	145.1	145.8	145.6	C_t-4a
148.8	148.7	148.8	148.3	C_t-8
174.3	174.3	174.3	174.3	C_l-1
185.5	185.5	185.5	185.4	C_t-6

[a]Shifts in parts per million downfield relative to tetramethylsilane.

[b]The numbering systems used are the standard numbering systems for the fatty acid chains and the α-tocopoherol moiety: C_l, the carbon atom in the fatty acid moiety; C_t, the carbon atom in the α-tocopherol moiety.

[c]Number of carbons in parentheses.

ν_{max} 2930, 1740, 1640, 1460, 1380, 1085, 920 cm^{-1}; MS *m/z* 445 (6%), 430 (10), 417 (3), 219 (2), 205 (5), 203 (12), 165 (17), 137 (9), 43 (100); $[\alpha]_D^{25}$ +14 (c 0.21, ethanol). A mixture of methyl 9(*S*)-[8a(*R*)-dioxy-α-tocopherone]-12(*S*), 13(*S*)-epoxy-10(*E*)-octadecenoate (**2d**) and methyl 11(*R*)-[8a(*S*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoate (**3b**) [the ratio of 9(*S*)- and 11(*R*)-isomers was about 2:1 based on the ^{1}H NMR signals]: IR (film) ν_{max} 2930, 1740, 1640, 1460, 1380, 1085, 965, 920 cm^{-1}; MS *m/z* 445 (6%), 430 (10), 417 (3), 219 (2), 205 (5), 203 (12), 165 (17), 137 (9), 43 (100); $[\alpha]_D^{25}$ − 31 (c 0.44, ethanol). **3c**, methyl 11(*S*)-[8a(*S*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoate: IR (film) ν_{max} 2930, 1740, 1640, 1460, 1380, 1085, 920 cm^{-1}: MS *m/z* 445 (5%), 430 (13), 417 (3), 219 (2), 205 (4), 203 (12), 165 (20), 137 (8), 43 (100); $[\alpha]_D^{25}$ + 14 (c 0.14, ethanol). **3d**, methyl 11(*R*)-[8a(*R*)-dioxy-α-tocopherone]-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoate: IR (film) ν_{max} 2930, 1740, 1640, 1460, 1380, 1085, 920 cm^{-1}; MS *m/z* 445 (5%), 430 (11), 417 (3), 219 (2), 205 (5), 203 (12), 165 (18), 137 (8), 43 (100); $[\alpha]_D^{25}$ − 38 (c 0.25, ethanol).

Compounds **4a** and **4b** were obtained as colorless oils (yields: **4a**, 54.8 mg; **4b**, 59.1 mg). After reduction and trimethylsilylation, each compound gave two major peaks corresponding to trimethylsilyl derivatives of methyl 13-hydroxy-9,11-octadecadienoate and TOH by the GC/MS analysis (9). The structures were identified by comparison with those of authentic samples as reported previously (11): compound **4a** was methyl 13(*S*)-[8a(*S*)-dioxy-α-tocopherone]-9(*Z*),11(*E*)-octadecadienoate and **4b** was methyl 13(*S*)-[8a(*R*)-dioxy-α-tocopherone]-9(*Z*),11(*E*)-octadecadienoate.

TOH was reacted with 13-MLOOH in the presence of Fe(III)AA in benzene under air. Figure 2 shows the results of TOH decay and the formation of the reaction products of

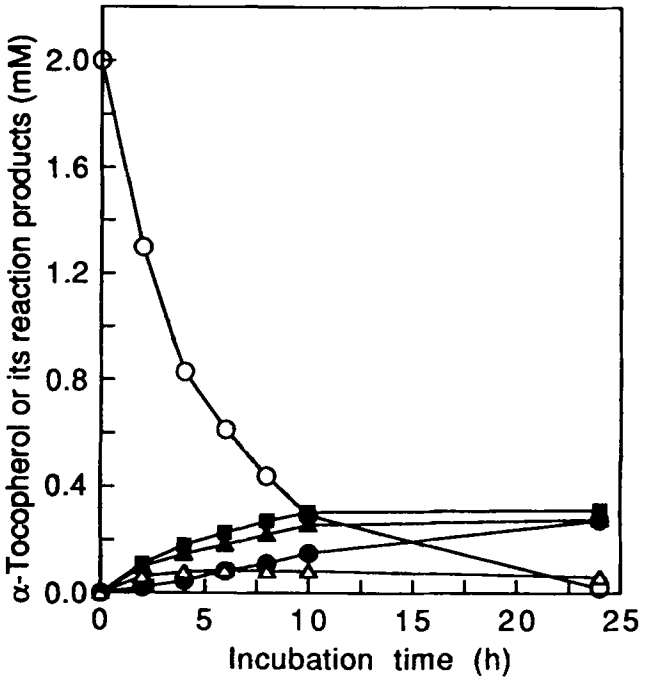


FIG. 2. Reaction of α-tocopherol during the Fe(III) acetylacetone-catalyzed [Fe(III)AA] decomposition of methyl 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate (13-MLOOH) under air. A reaction mixture containing 10 mM 13-MLOOH, 2.0 mM α-tocopherol and 2.0 mM Fe(III)AA in benzene was incubated at 37°C under air. Residual amounts of α-tocopherol (○) and the reaction products, compound **1** (●), compounds **2** and **3** (▲), compound **4** (■) and α-tocopherol dimer (△), in the reaction mixture are shown.

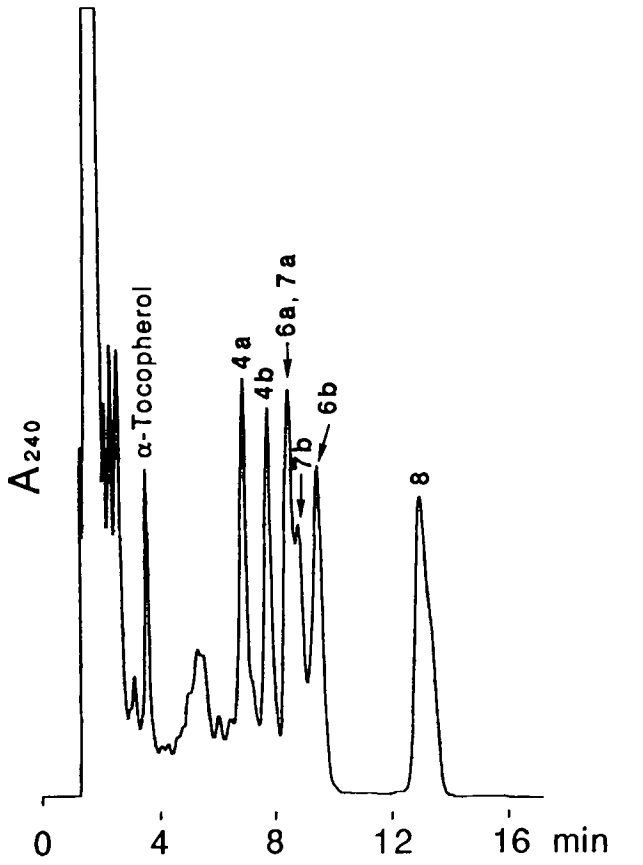
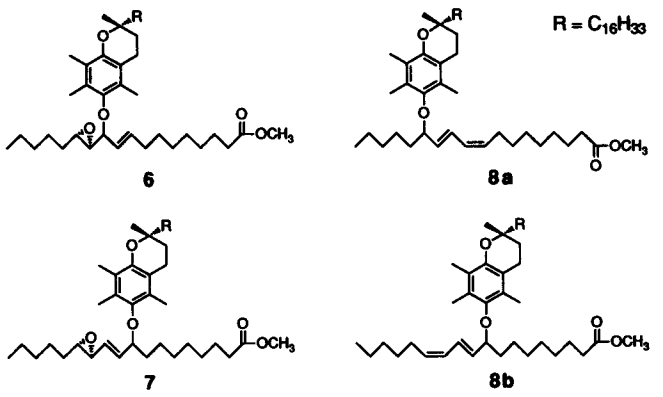


FIG. 3. Reversed-phase high-performance liquid chromatography (HPLC) of the products of the Fe(III)AA-catalyzed reaction of 13-MLOOH and α-tocopherol under nitrogen atmosphere for 20 h. HPLC was done with a μBondasphere 5μC_{18} column (3.9 × 150 mm) developed with methanol/ethanol (3:2, vol/vol) at 1.0 mL/min. Abbreviations and company location as in Figures 1 and 2.

TOH during the reaction. 8a-Epoxyalkylperoxy-α-tocopherones (**2** and **3**), 8a-alkylperoxy-α-tocopherones (**4**), and TOH dimer (**5**) formed initially, and then 4a,5-epoxy-8a-hydroperoxy-α-tocopherones (**1**) accumulated with the decrease of TOH. Compounds **1–5** accounted for 50% of the consumed TOH in the 10-h reaction mixture, and the rest were unknown products.

Reaction products under anaerobic conditions. Figure 3 shows the Fe(III)-catalyzed reaction products of TOH with 13-MLOOH under nitrogen atmosphere. Six peaks that appeared to be the reaction products of TOH were detected on the chromatogram. Peaks corresponding to compounds **4a** and **4b** were already obtained under the aerobic conditions as described previously. Each peak was collected by preparative reversed-phase HPLC. A peak containing compounds **6a** and **7a** were further separated by normal-phase HPLC using hexane/2-propanol (100:0.3, vol/vol), and **6a** and **7a** were eluted in that order. Thus, compounds **5–8** were obtained as colorless oils (yields: **6a**, 65.4 mg; **6b**, 103.9 mg; **7a**, 87.5 mg; **7b**, 47.3 mg; **8**, 27.9 mg), and their structures were identified as follows (Scheme 2).

The spectral data of **6a** and **6b** were consistent with those expected for methyl 11-(α-tocopheroxy)-12(*S*),13(*S*)-epoxy-9(*E*)-octadecenoates (13). Compounds **6a** and **6b** had the IR absorption at 970 cm^{-1} due to a *trans* double bond. The ^1H NMR spectra have signals at δ 2.57–3.03 due to methin protons on epoxy rings and the coupling constants (J = 2.2–2.4 Hz) show that the configuration of the epoxy groups is *trans* (13). From the coupling constants between the protons at C-11 (δ 3.81–3.87) and C-12 (δ 2.91–3.03) of the fatty acid moiety in **6a** (J = 6.2 Hz) and **6b** (J = 5.7 Hz), **6a** and **6b** appear to be the *threo-* and *erythro-*(α-tocopheroxy)-epoxides (13,19). Thus, the stereochemistry of **6a** and **6b** was assumed as follows: **6a**, methyl 11(*R*)-(α-tocopheroxy)-12(*S*),13(*S*)-epoxy-9(*E*)-octadecenoate: UV (ethanol) λ_{max} 282 (sh ε 2000), 289 nm (2300); IR (film) ν_{max} 2930, 1740, 1460, 1380, 1250, 1085, 970, 905 cm^{-1}; MS *m/z* 430 (62%), 205 (8), 203 (6), 189 (3), 177 (4), 175 (3), 165 (77), 151 (8), 121 (10), 43 (100); ^1H NMR ($CDCl_3$) δ 0.83–0.89 (*m*, 15H), 1.04–2.15 (*m*, 43H), 1.21 (*s*, 3H), 2.05 (*s*, 3H), 2.10 (*s*, 3H), 2.15 (*s*, 3H), 2.29 (*t*,J = 7.5 Hz, 2H), 2.54 (*t*,J = 6.4 Hz, 2H), 2.78 (*dt*,J = 2.2, 5.7 Hz, 1H), 3.03 (*dd*,J = 2.4, 6.2 Hz, 1H), 3.66 (*s*, 3H), 3.81 (*t*,J = 6.4 Hz, 1H), 5.52–5.56 (*m*, 2H); ^{13}C NMR ($CDCl_3$) δ 11.9, 12.9, 13.8, 14.0, 19.7, 19.8, 20.8, 21.1, 22.6 (2 atoms), 22.7, 23.8, 24.5, 24.8, 25.0, 25.7, 28.0, 28.8, 28.9, 29.1 (2 atoms), 31.5, 31.6, 31.7, 32.3, 32.7, 32.8, 34.1, 37.3, 37.5 (3 atoms), 39.4, 40.1, 51.4, 56.0, 60.5, 74.6, 84.6, 117.3,



SCHEME 2

122.6, 125.8, 126.2, 128.1, 135.4, 146.9, 147.6, 174.2; $[\alpha]_D^{25}$ – 21 (c 0.65, ethanol). **6b**, methyl 11(*R*)-(α-tocopheroxy)-12(*S*),13(*S*)-epoxy-9(*E*)-octadecenoate: UV (ethanol) λ_{max} 282 (sh ε 2010), 289 nm (2330); IR (film) ν_{max} 2930, 1740, 1460, 1380, 1250, 1085, 970, 905 cm^{-1}; MS *m/z* 430 (85%), 205 (10), 203 (6), 189 (2), 177 (4), 175 (2), 165 (86), 151 (9), 121 (9), 43 (100); ^1H NMR ($CDCl_3$) δ 0.83–0.88 (*m*, 15H), 1.04–2.26 (*m*, 43H), 1.22 (*s*, 3H), 2.06 (*s*, 3H), 2.09 (*s*, 3H), 2.13 (*s*, 3H), 2.29 (*t,J* = 7.4 Hz, 2H), 2.54 (*t,J* = 6.6 Hz, 2H), 2.57 (*m*, 1H), 2.91 (*dd,J* = 2.3, 5.8 Hz, 1H), 3.66 (*s*, 3H), 3.87 (*t,J* = 5.7 Hz, 1H), 5.61–5.63 (*m*, 2H); ^{13}C NMR ($CDCl_3$) δ 11.8, 12.9, 13.8, 14.0, 19.6, 19.7, 20.7, 21.0, 22.5, 22.6, 22.7, 23.7, 24.4, 24.8, 24.9, 25.4, 28.0, 28.8, 28.9, 29.0 (2 atoms), 31.5, 31.6 (2 atoms), 32.3, 32.7, 32.8, 34.0, 37.3, 37.5 (3 atoms), 39.4, 40.0, 51.3, 57.8, 58.6, 74.7, 84.3, 117.4, 122.7, 126.0, 126.2, 127.9, 135.7, 146.4, 147.6, 174.1; $[\alpha]_D^{25}$ – 1 (c 1.0, ethanol).

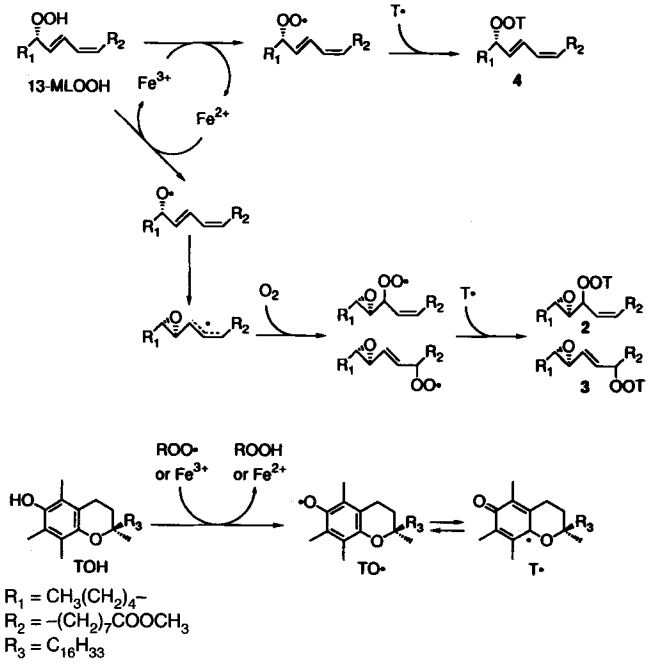
Compounds **7a** and **7b** were identified as stereoisomers of methyl 9-(α-tocopheroxy)-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoate from their spectral data. **7a**: UV (ethanol) λ_{max} 282 (sh ε 2060), 290 nm (2340); IR (film) ν_{max} 2930, 1740, 1460, 1380, 1250, 1085, 965, 885 cm^{-1}; MS *m/z* 430 (91%), 205 (9), 203 (7), 189 (3), 177 (4), 175 (3), 165 (92), 151 (11), 121 (11), 43 (100); ^1H NMR ($CDCl_3$) δ 0.83–0.91 (*m*, 15H), 1.07–2.11 (*m*, 43H), 1.22 (*s*, 3H), 2.06 (*s*, 6H), 2.11 (*s*, 3H), 2.29 (*t,J* = 7.5 Hz, 2H), 2.54 (*t,J* = 6.3 Hz, 2H), 2.65 (*dt,J* = 2.1, 5.2 Hz, 1H), 3.01 (*dd,J* = 1.9, 7.1 Hz, 1H), 3.66 (*s*, 3H), 4.04 (*dt,J* = 5.5, 7.9 Hz, 1H), 5.20 (*dd,J* = 7.2, 15.5 Hz, 1H), 5.89 (*dd,J* = 8.1, 15.6 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.9, 13.2, 14.0 (2 atoms), 19.7, 19.8, 20.8, 21.1, 22.6 (2 atoms), 22.7, 23.8, 24.5, 24.8, 24.9, 25.3, 25.5, 28.0, 29.1, 29.2, 29.5, 31.5, 31.6, 31.9, 32.7, 32.8, 34.0, 34.9, 37.3, 37.4, 37.5 (2 atoms), 39.4, 40.0, 51.4, 57.6, 60.5, 74.6, 82.8, 117.3, 122.6, 126.2, 128.1, 130.3, 134.2, 146.8, 147.4, 174.2; $[\alpha]_D^{25}$ + 9 (c 0.88, ethanol). **7b**: UV (ethanol) λ_{max} 282 (sh ε 2110), 290 nm (2400); IR (film) ν_{max} 2930, 1740, 1460, 1380, 1250, 1085, 965, 885 cm^{-1}; MS *m/z* 430 (92%), 205 (11), 203 (7), 189 (3), 177 (4), 175 (3), 165 (100), 151 (13), 121 (11), 43 (98); ^1H NMR ($CDDl_3$) δ 0.83–0.92 (*m*, 15H), 1.04–2.10 (*m*, 43H), 1.22 (*s*, 3H), 2.06 (*s*, 6H), 2.10 (*s*, 3H), 2.30 (*t,J* = 7.5 Hz, 2H), 2.54 (*t,J* = 6.7 Hz, 2H), 2.63 (*dt,J* = 2.2, 5.4 Hz, 1H), 3.01 (*dd,J* = 2.2, 7.9 Hz, 1H), 3.66 (*s*, 3H), 4.01 (*dt,J* = 5.4, 8.0 Hz, 1H), 5.07 (*dd,J* = 7.9, 15.7 Hz, 1H), 5.89 (*dd,J* = 8.1, 15.6 Hz, 1H); ^{13}C NMR ($CDDl_3$) δ 11.9, 13.2, 14.0 (2 atoms), 19.7, 19.8, 20.8, 21.1, 22.6 (2 atoms), 22.7, 23.7, 24.5, 24.8, 24.9, 25.3, 25.6, 28.0, 29.1, 29.2, 29.5, 31.5, 31.6, 32.0, 32.7, 32.8, 34.1, 34.9, 37.3, 37.5 (3 atoms), 39.4, 40.1, 51.4, 57.9, 60.4, 74.7, 83.0, 117.3, 122.6, 126.3, 128.2, 131.1, 134.5, 146.7, 147.4, 174.3; $[\alpha]_D^{25}$ – 27 (c 0.95, ethanol). It is obvious that the configuration of the α-tocopheroxy group in **7** is responsible for the structural difference between **7a** and **7b**. Furthermore, the CD spectra of **7a** and **7b** in ethanol showed a positive and a negative Cotton effect, respectively, indicating that these compounds are optical isomers (data not shown). However, the stereochemistry of **7a** and **7b** could not be assigned.

Compound **8** was identified by comparison of the HPLC behavior with those of authentic samples as reported previously (11). Compound **8** contained methyl 13-(α-tocopheroxy)-9(*Z*),11(*E*)-octadecadienoate (**8a**) and methyl 9-(α-tocopheroxy)-10(*E*),12(*Z*)-octadecadienoate (**8b**). We did not study the time course experiment under nitrogen atmosphere, because contamination with air in the reaction system could not be prevented.

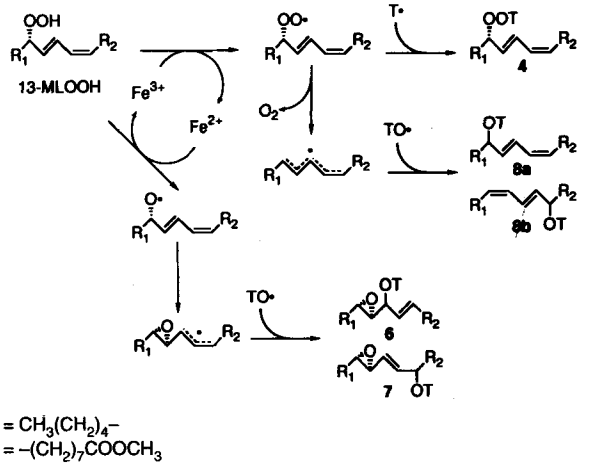
DISCUSSION

TOH reacts with a variety of free radicals and forms various reaction products. In the present study, we have isolated and characterized the reaction products of TOH with 13-MLOOH in the presence of Fe(III)AA. The main products under air were 8a-(lipid-peroxy)-α-tocopherones (**2–4**) in addition to 4a,5-epoxy-8a-hydroperoxy-α-tocopherones (**1**) and TOH dimer (**5**). Gruger and Tappel (21) and Igarashi *et al.* (22) reported that α-tocopheryl quinone was formed during the Fe(III)-catalyzed reactions between TOH and MLOOH. Gardner *et al.* (12) also obtained α-tocopheryl quinone as the Fe(III)-catalyzed reaction products of LOOH and TOH in the presence of air. The formation of α-tocopheryl quinone is undoubtedly due to hydrolysis of the 8a-(lipid-peroxy)-α-tocopherones. The 8a-(lipid-peroxy)-α-tocopherones are unstable in acid (23), and the chromatographic separation or the acidic condition may result in the formation of α-tocopheryl quinone. The reaction products under nitrogen atmosphere were almost the same as those reported by Kaneko and Matsuo (13) and Gardner (24). The main products were the addition compounds of α-tocopheroxyl radical with the epoxyalkyl radicals (**6** and **7**). In addition to these 6-*O*-epoxyalkyl-α-tocopherones, we also detected 8a-(lipid-peroxy)-α-tocopherones (**4**) and 6-*O*-alkyl-α-tocopherols (**8**).

The Fe(III)AA-catalyzed decomposition of 13-MLOOH and the reaction with TOH in homogeneous solution at sufficient oxygen pressure are illustrated in Scheme 3. The first reaction would be iron-dependent free radical formation by being oxidized 13-MLOOH to a peroxyl radical or reduced to an alkoxyl radical (1,2). It is also likely that the Fe(III)AA reacts very readily with TOH to give Fe(II)AA and α-toco-



SCHEME 3



SCHEME 4

pheroxyl radical (12). This assertion is supported by the observation of significant amounts of TOH dimer (**5**) derived from the bimolecular self-reaction of α-tocopheroxyl radical, consistent with the presence of a high concentration of α-tocopheroxyl radical (25). Furthermore, TOH can trap the peroxyl radical by hydrogen-atom transfer, giving a hydroperoxide and an α-tocopheroxyl radical (3). The alkoxyl radical which was produced by the Fe(II)AA-catalyzed decomposition of 13-MLOOH then adds to the adjacent double bond to form the carbon-centered 12,13-epoxyalkyl radical (26–28). In the presence of oxygen, the epoxyalkyl radical reacts with molecular oxygen to form peroxyl radicals. Coupling of oxygen to the carbon-centered radical occurs at a diffusion-controlled rate at ambient oxygen concentration (29). The α-tocopheroxyl radical reacts rapidly and irreversibly with these peroxyl radicals to form stable products (11). In the present study, the 13(*S*)-peroxyl radical reacted with α-tocopheroxyl radical to form methyl 13(*S*)-(8a-dioxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoates (**4**); the 12,13-epoxy-9-peroxyl and the 12,13-epoxy-11-peroxyl radicals reacted with α-tocopheroxyl radical to form methyl 9-(8a-dioxy-α-tocopherone)-12(*S*),13(*S*)-epoxy-10(*E*)-octadecenoates (**2**) and methyl 11-(8a-dioxy-α-tocopherone)-12(*S*),13(*S*)-epoxy-9(*Z*)-octadecenoates (**3**), respectively.

There may be another pathway for the reaction of α-tocopheroxyl radical. The pathway is thought to be the formation of 4a,5-epoxy-8a-hydroperoxy-α-tocopherone (**1**) by peroxyl-radical dependent epoxidation of α-tocopheroxyl radical and oxygen addition to the 8a-position followed by hydrogen addition (7,30). However, this reaction pathway is still unclear.

All the epoxyalkyl radicals produced are expected to react very rapidly with oxygen. When the oxygen pressure is lowered, the epoxyalkyl radical can directly react with the phenoxyl radical of TOH (12,13,28). Scheme 4 shows the Fe(III)AA-catalyzed reaction mechanisms of α-tocopheroxyl radical with 13-MLOOH under anaerobic conditions. The carbon-centered 12,13-epoxyalkyl radicals from 13-MLOOH could attack the α-tocopheroxyl radical to form the 6-*O*-epoxyalkyl-α-tocopherols (**6** and **7**) under nitrogen atmosphere. In addition, radical elimination of the peroxyl radical may result in the formation of an alkyl radical and oxygen (31). The formation of the carbon-centered alkyl radical is substantiated by the finding that the isomerization of methyl linoleate hydroperoxides is accompanied by the exchange of the oxygen atoms of the hydroperoxy group with atmospheric oxygen (32). The alkyl radical produced then reacted with α-tocopheroxyl radical to form 6-*O*-alkyl-α-tocopherols (**8**) (11). Although the 6-*O*-alkyl-α-tocopherol was produced in low yields, the elimination reaction of peroxyl radicals might occur under the anaerobic conditions.

The reaction products obtained in the present study indicate that peroxyl and oxygen radicals prefer to react with the 8a-carbon radical of TOH; whereas, the phenoxyl radical of TOH appears to react with carbon radicals of fatty acids.

ACKNOWLEDGMENTS

We thank H. Inoue for recording the NMR spectra. This study was supported in part by the Asahi Breweries Foundation, Tokyo, Japan.

REFERENCES

1. Halliwell, B., and Gutteridge, M.C. (1984) *Biochem. J. 219*, 1–14.
2. Sevanian, A., and Hochstein, P. (1985) *Ann. Rev. Nutr. 5*, 365–390.
3. Burton, G.W., and Ingold, K.U. (1986) *Acc. Chem. Res. 19*, 194–201.
4. Nilsson, J.L.G., Daves, G.D., Jr., and Folkers, K. (1968) *Acta Chem. Scand. 22*, 207–218.

5. Fujimaki, M., Kanamaru, K., Kurata, T., and Igarashi, O. (1970) *Agric. Biol. Chem. 34*, 1781–1786.
6. Winterle, J., Dulin, D., and Mill, T. (1984) *J. Org. Chem. 49*, 491–495.
7. Matsuo, M., Matsumoto, S., Iitaka, Y., and Niki, E. (1989) *J. Am. Chem. Soc. 111*, 7179–7185.
8. Yamauchi, R., Matsui, T., Kato, K., and Ueno, Y. (1989) *Lipids 24*, 204–209.
9. Yamauchi, R., Matsui, T., Kato, K., and Ueno, Y. (1990) *Lipids 25*, 152–158.
10. Liebler, D.C., Baker, P.F., and Kaysen, K.L. (1990) *J. Am. Chem. Soc. 112*, 6995–7000.
11. Yamauchi, R., Miyake, N., Kato, K., and Ueno, Y. (1993) *Lipids 28*, 201–206.
12. Gardner, H.W., Eskins, K., Grams, G.W., and Inglett, G.E. (1972) *Lipids 7*, 324–334.
13. Kaneko, T., and Matsuo, M. (1985) *Chem. Pharm. Bull. 33*, 1899–1905.
14. Gardner, H.W. (1975) *Lipids 10*, 248–252.
15. Chan, H.W.-S., and Levett, G. (1977) *Lipids 12*, 99–104.
16. Yamauchi, R., Kato, K., and Ueno, Y. (1988) *Lipids 23*, 779–783.
17. Oette, K. (1965) *J. Lipid Res. 6*, 449–454.
18. Hamberg, M. (1975) *Lipids 10*, 87–92.
19. Gardner, H.W., and Kleiman, R. (1981) *Biochim. Biophys. Acta 665*, 113–125.
20. Coxon, D.T., Price, K.R., and Chan, H.W.-S. (1981) *Chem. Phys. Lipids 28*, 365–378.
21. Gruger, E.H., Jr., and Tappel, A.L. (1970) *Lipids 5*, 326–331.
22. Igarashi, O., Matsukawa, H., and Inagaki, C. (1976) *J. Nutr. Sci. Vitaminol. 22*, 267–270.
23. Liebler, D.C., Kaysen, K.L., and Kennedy, T.A. (1989) *Biochemistry 28*, 9772–9777.
24. Gardner, H.W. (1983) in *Xenobiotics in Foods and Feeds* (Finley, J.W., and Schwass, D.E., eds.) ACS Symposium Series 234, pp. 63–84, American Chemists' Society, Washington, D.C.
25. Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L., and Ingold, K.U. (1985) *J. Am. Chem. Soc. 107*, 7053–7065.
26. Dix, T.A., and Marnett, L.J. (1981) *J. Am. Chem. Soc. 103*, 6744–6746.
27. Gardner, H.W. (1989) *Free Radical Biol. Med. 7*, 65–86.
28. Wilcox, A.L., and Marnett, L.J. (1993) *Chem. Res. Toxicol. 6*, 413–416.
29. Erben-Russ, M., Bors, W., and Saran, M. (1987) *Int. J. Radiat. Biol. 52*, 393–412.
30. Liebler, D.C., Kaysen, K.L., and Burr, J.A. (1991) *Chem. Res. Toxicol. 4*, 89–93.
31. Porter, N.A., and Wujek, D.G. (1984) *J. Am. Chem. Soc. 106*, 2626–2629.
32. Chan, H.W.-S., Levett, G., and Matthew, J.A. (1978) *J. Chem. Soc. Chem. Commun.*, 756–757.

[Received September 22, 1993, and in revised form February 9, 1995; Revision accepted February 16, 1995]

Oxidized Cholesterol Modulates Age-Related Change in Lipid Metabolism in Rats

Kyoichi Osada, Takehiro Kodama, Satoshi Noda, Koji Yamada and Michihiro Sugano*

Laboratory of Food Science, Kyushu University, School of Agriculture, Fukuoka 812, Japan

ABSTRACT: For three weeks, male Sprague-Dawley rats at either four weeks (young) or eight months (adult) of age were pair-fed one of the purified diets free of or containing either 0.2% of oxidized cholesterol mixture (cholesterol oxidation products) or 0.2% of cholesterol. Although the food intake was similar, dietary oxidized cholesterol lowered body weight gain in young rats, but did not increase relative liver weight, in contrast to the enlargement seen with dietary cholesterol. Oxidized cholesterol, compared to cholesterol, tended to reduce the activity of hepatic 3-hydroxy-3-methylglutaryl CoA reductase and cholesterol 7α-hydroxylase, particularly the latter in aged rats, and prevented the rise in the concentration of liver cholesterol at both ages. It also tended to increase the activity of hepatic Δ6 desaturase, particularly in young rats. Moreover, oxidized cholesterol in relation to cholesterol influenced liver and serum lipid concentrations in different ways, and increased lipid peroxidation at both ages. The ratio of splenic $CD4^+/CD8^+$ T-lymphocytes increased with age, but the influence of cholesterol and oxidized cholesterol was comparable. Thus, oxidized cholesterol may specifically disturb growth and age-related changes in the lipid metabolism in rats.

Lipids 30, 405–413 (1995).

Lipid oxidation products are present in varying amounts in processed foods. It is well understood that these oxidized lipids show various undesirable effects, such as the acceleration of the atherogenic process (1). Among oxidized lipids, cholesterol autoxidation products (oxidized cholesterol) have received much attention in recent years with respect to their diverse biological activities: carcinogenicity (2), cytotoxicity (3), mutagenicity (4), and inhibition of sterogenesis (5). However, these observations are based exclusively on the *in vitro* studies and have not been studied in depth with respect to the situation *in vivo*. Substantial amounts of oxidized cholesterol are detected in a variety of foods of animal origin exposed to oxidizing conditions (6–10). We have reported previously that oxidized cholesterol in the diet was absorbed in detectable amounts, around 30% of that ingested (11), and disturbed cholesterol and linoleic acid metabolism (12).

In addition to these observations, Emanuel *et al.* (13) showed that increased dietary oxidized cholesterol led to increased concentrations in human serum, and Cho *et al.* (14) reported that oxidized cholesterol disturbs the biosynthesis of cholesterol in chicks. However, the effect of oxidized cholesterol on the aging process was not mentioned, despite the possibility that the changes associated with aging are promoted by lipid peroxidation (15). Therefore, it is of interest to know the effect of dietary oxidized cholesterol on this metabolic process, particularly in relation to the modification of lipid metabolism.

Choi *et al.* (16) showed the reduction of the key enzymes in cholesterol metabolism in aged rats. The age-related change in cholesterol metabolism in rats has been shown to be largely completed by the age of 8–9 mon, the additional changes after that being rather marginal (17). Therefore, we studied the effect of oxidized cholesterol on the metabolism of cholesterol and fatty acid using young (four-week-old) and adult (eight-month-old) rats to determine age-related differences using a pair-feeding protocol.

Recently, Hobbs *et al.* (18) suggested that gradual age-associated shifts in the subset composition of the splenic $CD4^+$ cell pool underlie progressive changes in the patterns of cytokine gene expression by this cell group. However, the relationship among diets, aging, and splenic T-cell subsets has not been fully explored. Therefore, the interaction of oxidized cholesterol and aging on T-cell subsets was also studied.

MATERIALS AND METHODS

Materials. 3-Hydroxy-3-methyl[1-^{14}C]glutaryl CoA (55 mCi/mmol) was obtained from Amersham International plc (Buckinghamshire, United Kingdom). [1-^{14}C]Linoleic acid (53 mCi/mmol) and [4-^{14}C]cholesterol (51 mCi/mmol) were the products of New England Nuclear (Boston, MA). Radioactive cholesterol and linoleic acid were purified by thin-layer chromatography prior to use. All reagents used were analytical grade.

Animals and diets. Young (four-week-old) and adult (eight-month-old) male Sprague-Dawley rats obtained from Seiwa Experimental Animals (Fukuoka, Japan) were housed individually in a room with controlled temperature and light

*To whom correspondence should be addressed at the Laboratory of Food Science, Department of Food Science and Technology, Faculty of Agriculture 46-09, Kyushu University, Higashi-ku, Fukuoka 812, Japan.

Abbreviations: ACAT, acyl-CoA:cholesterol *O*-acyltransferase; FBS, fetal bovine serum; GLC, gas–liquid chromatography; GPT, glutamic pyruvic transaminase; HMG–CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TBA, thiobarbituric acid.

Copyright © 1995 by AOCS Press

(20–23°C and lights on from 0800 to 2000). After acclimation for four days, the rats were divided into groups of six each and fed one of the cholesterol-free, cholesterol-added, or oxidized cholesterol-added diets. The cholesterol-free and cholesterol-added groups were fed diets at the level consumed by the oxidized cholesterol group. After three weeks, rats were decapitated at night (0100). Feces were collected for two days beginning 10 d before killing, and then lyophilized. Experimental diets were prepared according to the formula recommended by the American Institute of Nutrition (19), and contained (wt%): casein, 20; safflower oil (Rinoru Oil Co., Nagoya, Japan), 5; mineral mixture, 3.5; vitamin mixture, 1.0; choline bitartrate, 0.2; DL-methionine, 0.3; cellulose, 5.0; corn starch, 15; sodium cholate, 0.05; and sucrose to 100. Cholesterol (0.2%) and the oxidized cholesterol mixture (0.2%) were added at the expense of sucrose. Food was changed daily, and food intake and weight gain were recorded.

Preparation of oxidized cholesterol mixture (cholesterol oxidation products). Cholesterol (300 g) was heated in an electric oven at 150°C for 12 h. Heated cholesterol was dissolved in *n*-hexane, applied on a silicic acid column (Silica gel 60, 70–230 mesh; E. Merck, Darmstadt, Germany; 100 ϕ mm × 800 mm) and fractionated by successive elution with 1 L each of *n*-hexane, diethyl ether, and methanol. An aliquot of the polar fraction eluted by methanol was dried in a rotary evaporator and finally *in vacuo*. The composition of the oxidized cholesterol mixture is shown in Table 1. The preparation contained more than 90% as oxidized cholesterol.

Analyses. The activities of 3-hydroxy-3-methylglutaryl (HMG)–CoA reductase, cholesterol 7α-hydroxylase, and Δ6 desaturase in liver microsomes were measured by the methods of Shapiro *et al.* (20), Van Cantfort *et al.* (21), and Svensson (22), respectively. Microsomal protein was determined by the method of Lowry *et al.* (23). Serum and liver lipids were extracted, and cholesterol, triglyceride, and phospholipid were measured (24). The fatty acid composition of liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was analyzed by gas–liquid chromatography (GLC) (25). Fecal neutral and acidic steroids were also analyzed by GLC using 5α-cholestane (Sigma Chemical Co., St. Louis, MO) and 23-nor-deoxycholic acid (Steraloids Inc., Wilton, NH) as internal standards, respectively (26). During the feeding period, blood was withdrawn from the tail vein on days 0, 5, 10, and 15, and serum cholesterol was determined enzymatically (Cholesterol C Test, Wako Pure Chemicals Co., Osaka, Japan). Liver and blood lipoperoxides were measured by the method of Yagi and co-workers (27,28) as thiobarbituric acid (TBA) reactive substances. Oxidized cholesterol contents of liver and serum were measured by capillary GLC on fused silica capillary ULBON HR-1 column (Shinwa Chemical Industries, Ltd., Kyoto, Japan) under the conditions described previously (10). The serum glutamic pyruvic transaminase (GPT) activity also was measured by the enzymatic method (GPT-UV Test; Wako Pure Chemicals Co.).

Separation of spleen lymphocytes analysis of $CD4^+$ and $CD8^+$ cells. Lymphocytes were squeezed out into the RPMI 1640 medium from isolated spleen using glass slides. After incubating the cells at 37°C for 30 min to remove fibroblasts, 5 mL of the cell suspension was layered on 4 mL of Lymphocyte-Rat (Cedarlane, Hornby, United Kingdom) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed three times with the RPMI 1640 medium. The lymphocytes were resuspended in the phosphate-buffered saline (PBS; pH 7.5) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). The cell concentration was then adjusted to 1×10^6 cells/100 μL, and 5 μL of either fluorescein-labeled mouse anti-rat CD4 or phycoerythrin-labeled mouse anti-rat CD8 (Serotec Ltd., Kidlington, Oxford, United Kingdom) were added. After incubation for 30 min at 4°C, lymphocytes were rinsed three times with PBS containing 10% FBS and then centrifuged at 1,200 rpm for 5 min. The stained lymphocytes were fixed with 2% paraformaldehyde and applied to flow cytometry (EPICS PLOFILE II; Coulter Electronics Ltd., Luto, Beds, United Kingdom).

Statistical analysis. Data were analyzed by Student's *t*-test (29) and Duncan's new multiple-range test (30) to determine the exact nature of the differences among the groups. The interaction of the age and sterol was evaluated by two-way analysis of variance (31).

TABLE 1
Composition of Cholesterol Oxidation Products

Components	Wt%
7α-Hydroxycholesterol	6.0
Cholesterol	8.0
7β-Hydroxycholesterol	13.3
5β-Epoxycholesterol	3.9
5α-Epoxycholesterol	14.2
Cholestanetriol	3.3
7-Ketocholesterol	26.7
25-Hydroxycholesterol	1.7
Unknown steroids[a]	18.5
Total oxidized cholesterol	92.0

[a]Composed of more than 20 components.

RESULTS

Growth and liver weight. Although the food intake was adjusted to be the same by pair-feeding, weight gain in young, but not in adult rats fed oxidized cholesterol was significantly lower than in the other two groups (Table 2). Oxidized cholesterol, in contrast to cholesterol, did not increase relative liver weight in both ages.

Concentration of serum and liver lipids. The time course of the changes in serum cholesterol concentrations is shown in Figure 1. In young rats, the serum cholesterol levels were significantly higher in the cholesterol group than in the cholesterol-free and oxidized cholesterol groups throughout the feeding periods. In contrast, the levels were similar among the groups in adult rats, although the oxidized cholesterol group showed a slightly lower level than other groups.

TABLE 2
Effect of Dietary Cholesterol and Oxidized Cholesterol on Growth Parameters in Young and Adult Rats[a]

	Initial body wt (g)	Weight gain (g/21 d)	Food intake (g/d)	Liver weight (g/100 g B.W.)
Young				
Cholesterol-free	130 ± 3	147 ± 6[c,*]	20.6 ± 0.3*	4.93 ± 0.21[c,*]
Cholesterol	130 ± 3	146 ± 4[c,*]	21.0 ± 0.2*	5.73 ± 0.26[d,*]
Oxidized cholesterol	130 ± 4	78 ± 6[d,*]	20.4 ± 0.5*	4.69 ± 0.12[c,*]
Adult				
Cholesterol-free	667 ± 12	27 ± 12	25.0 ± 1.4	3.78 ± 0.17
Cholesterol	668 ± 15	24 ± 5	26.6 ± 0.7	4.12 ± 0.18
Oxidized cholesterol	668 ± 15	24 ± 8	26.2 ± 0.5	3.68 ± 0.20
ANOVA *f* value[b]				
Age	—	$P < 0.01$	$P < 0.01$	$P < 0.01$
Sterol	—	NS	NS	$P < 0.01$
Interaction	—	NS	NS	NS

[a]Data are presented as means ± SE for six rats in each group; B.W., body weight.
[b]Data were analyzed by two-way analysis of variance (ANOVA). When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test.
[c,d]Values without a common superscript letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult age rats at $P < 0.05$. NS, not significant.

As shown in Figure 2, when the diet containing oxidized cholesterol was fed, the concentration of liver cholesterol was significantly lower than when cholesterol was fed at both ages, and it was as low as that in the cholesterol-free group. The same response was observed in the concentration of serum cholesterol in young, but not in adult, rats. The concentration of liver triglyceride remained unchanged after feeding oxidized cholesterol and tended to be lower than in the cholesterol-free diet, particularly in young rats. There was no significant difference in the serum triglyceride concentration at either age. On the other hand, oxidized cholesterol tended to increase the liver phospholipid concentration in young rats compared to cholesterol. Both cholesterol and oxidized cholesterol increased serum phospholipid in young rats, while decreasing it in adult rats.

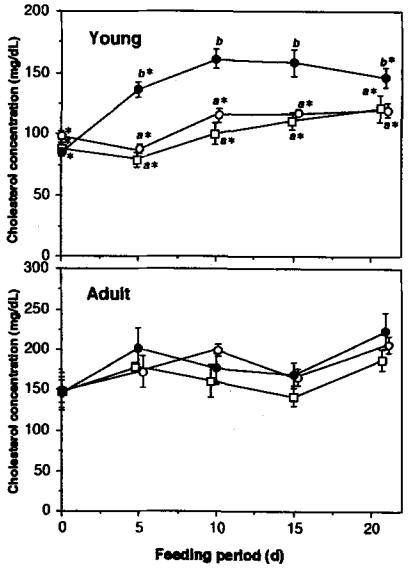


FIG. 1. Time course of the effect of dietary cholesterol and oxidized cholesterol on the concentration of serum cholesterol in young and adult rats. Data are presented as means ± SE for six rats in each group. ○, Cholesterol-free; ●, cholesterol; □, oxidized cholesterol. [a,b]Values in the same age group without a common letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult rats at $P < 0.05$.

Key enzyme activities of liver microsomes. Figure 3 shows the specific activities of HMG–CoA reductase, cholesterol 7α-hydroxylase, and Δ6 desaturase of liver microsomes. In young rats, the HMG–CoA reductase activity was significantly lower in the cholesterol group than in the cholesterol-free group, but this was not the case in adult rats. Oxidized cholesterol tended to reduce the reductase activity more than cholesterol in both ages, particularly in adult rats. Although dietary cholesterol tended to increase cholesterol 7α-hydroxylase activity, oxidized cholesterol reduced it in both age groups. There was a tendency to decrease the activity of Δ6 desaturase when cholesterol was fed compared to the cholesterol-free diet, particularly in young rats, whereas no such reduction was observed in the oxidized cholesterol group. The desaturase activities were higher in the oxidized cholesterol groups than in the cholesterol-free groups for both age groups, although they were not statistically significant.

Fatty acid compositions of liver phospholipids. As shown in Table 3, the significant diet-dependent difference was observed in the fatty acid composition of liver phospholipids. Dietary cholesterol increased the proportion of oleic and linoleic acids in PC and PE both in young and adult rats, whereas the proportion of arachidonic acid tended to decrease except for PE of young rats. As a result, the ratio of linoleic acid to its metabolites, (20:3n-6 + 20:4n-6)/18:2n-6, was significantly decreased in cholesterol-fed rats as compared to

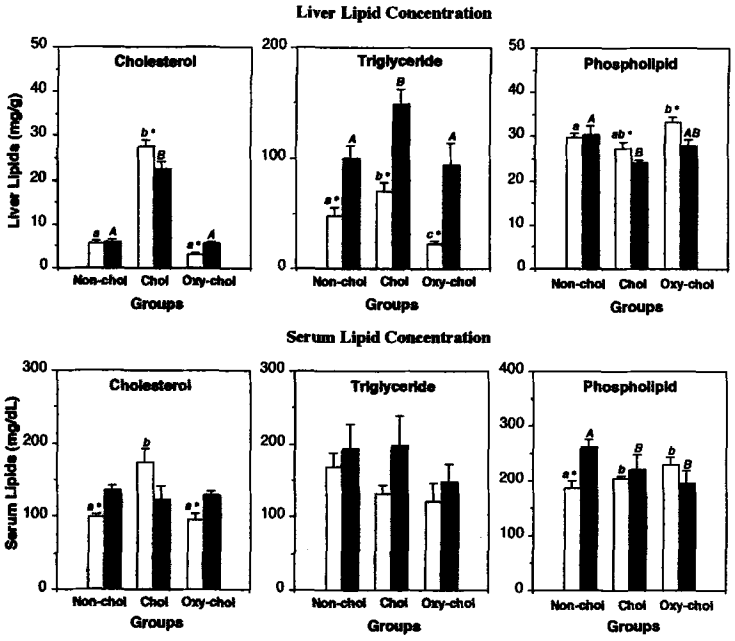


FIG. 2. Effect of dietary cholesterol and oxidized cholesterol on liver and serum lipid concentrations in young and adult rats. Data are presented as means ± SE for six rats in each group. □, Young; ■, adult. Data were analyzed by two-way analysis of variance. When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test. ^{a,b,c,A,B}Values without a common letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult rats at $P < 0.05$. Non-chol, cholesterol-free; Chol, cholesterol; Oxy-chol, oxidized cholesterol; NS, not significant. Analysis of variance: A = significant effect of age; S = significant effect of sterol; A × S = significant effect of interaction. Following significant difference was observed: liver cholesterol, S and A × S; liver triglyceride, A and S; liver phospholipid, A and S. Serum cholesterol, S and A × S, serum phospholipid, A and S.

those fed the cholesterol-free diet. In contrast, the ratio was significantly higher in the oxidized cholesterol group than in the cholesterol-free group, except for PC of young rats.

Fecal steroid excretion. As summarized in Table 4, there was no significant difference in the weight of dried feces among the same age groups. The excretion of total neutral steroid was at a similar level between the cholesterol and oxidized cholesterol groups in the respective age groups, and it was significantly higher than in the cholesterol-free group. The effect of oxidized cholesterol on the composition of fecal neutral steroids differed from that of cholesterol, and oxidized cholesterol significantly raised the excretion of coprostanol in young rats. Cholesterol significantly increased the excretion of acidic steroids as compared to the cholesterol-free group

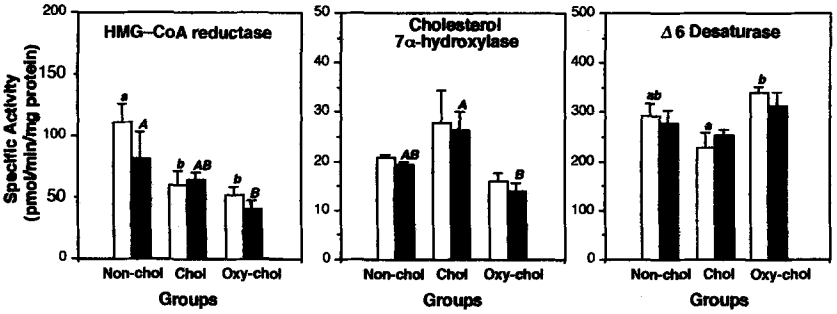


FIG. 3. Effect of dietary cholesterol and oxidized cholesterol on the activities of key enzymes in cholesterol metabolism and fatty acid desaturation in young and adult rats. Data are presented as means ± SE for six rats in each group. □, Young; ■, adult. Data were analyzed by two-way analysis of variance. When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test. ^{a,b,A,B}Values without a common superscript letter are significantly different at $P < 0.05$. Abbreviations as in Figure 2. The following significant difference was observed: 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, S; cholesterol 7α-hydroxylase, S; Δ6 desaturase, S.

TABLE 3
Effect of Dietary Cholesterol and Oxidized Cholesterol on Fatty Acid Compositions of Liver Phospholipids in Young and Adult Rats[a]

	Fatty acids (wt%)								
Groups	16:0	16:1	18:0	18:1	18:2n-6	20:3n-6	20:4n-6	22:6n-3	20:3 + 20:4/18:2
Phosphatidylcholine									
Young									
Cholesterol-free	19.5 ± 0.0*	2.2 ± 0.2^{c}	16.6 ± 0.6^{c,d,*}	9.1 ± 0.4^{c}	13.9 ± 0.8^{c}	1.3 ± 0.3^{c}	28.7 ± 1.3^{c}	2.5 ± 0.1^{c,*}	2.2 ± 0.2^{c}
Cholesterol	19.3 ± 0.2*	3.6 ± 0.1^{d}	14.7 ± 0.5^{c,*}	12.3 ± 0.2^{d,*}	17.7 ± 1.3^{d}	2.9 ± 0.5^{d}	23.2 ± 1.5^{d}	1.7 ± 0.2^{d,*}	1.5 ± 0.2^{d}
Oxidized cholesterol	18.6 ± 0.5	2.1 ± 0.2^{c,*}	18.1 ± 0.8^{d}	8.4 ± 0.4^{c}	11.1 ± 0.3^{c}	1.4 ± 0.4^{c}	28.7 ± 0.4^{c,*}	2.4 ± 0.1^{c,*}	2.7 ± 0.1^{c,*}
Adult									
Cholesterol-free	18.8 ± 0.4	2.8 ± 0.1^{g}	19.2 ± 0.6	8.7 ± 0.3^{g}	12.6 ± 0.6^{g}	1.6 ± 0.2^{g}	28.3 ± 0.6^{g}	5.9 ± 0.3^{g}	2.4 ± 0.2^{g}
Cholesterol	18.2 ± 0.4	3.8 ± 0.1^{h}	17.5 ± 0.7	10.8 ± 0.4^{h}	17.7 ± 1.2^{h}	3.4 ± 0.7^{h}	22.5 ± 0.6^{h}	3.9 ± 0.3^{h}	1.5 ± 0.2^{h}
Oxidized cholesterol	17.6 ± 0.7	2.7 ± 0.2^{g}	19.2 ± 0.9	9.1 ± 0.5^{g}	11.1 ± 0.6^{g}	1.5 ± 0.2^{g}	31.0 ± 0.6^{g}	5.8 ± 0.2^{g}	3.0 ± 0.1^{i}
ANOVA *f* value[b]									
Age	$P < 0.05$	$P < 0.01$	$P < 0.01$	NS	NS	NS	NS	$P < 0.01$	NS
Sterol	NS	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Interaction	NS	NS	NS	$P < 0.05$	NS	NS	NS	$P < 0.05$	NS
Phosphatidylethanolamine									
Young									
Cholesterol-free	21.2 ± 0.9^{c}	0.8 ± 0.2^{c}	21.2 ± 1.1^{c,*}	6.5 ± 0.2^{c}	3.4 ± 0.4^{c}	0.4 ± 0.1^{c}	30.3 ± 1.0^{c}	7.3 ± 1.0^{c,*}	8.4 ± 0.5^{c}
Cholesterol	18.1 ± 0.3^{d}	1.5 ± 0.2^{d}	17.0 ± 0.6^{d}	9.3 ± 0.5^{d,*}	5.8 ± 0.5^{d}	1.4 ± 0.2^{d}	34.9 ± 0.7^{d,*}	4.7 ± 0.5^{d,*}	6.3 ± 0.5^{d}
Oxidized cholesterol	19.6 ± 0.9^{c,d,*}	0.5 ± 0.2^{d}	18.9 ± 0.8^{c,d}	5.8 ± 0.4^{c,*}	3.0 ± 0.2^{c}	0.4 ± 0.1^{c}	32.4 ± 0.6^{c}	8.2 ± 1.8^{c,*}	11.2 ± 0.7^{e}
Adult									
Cholesterol-free	19.6 ± 0.6^{g}	1.4 ± 0.4^{g}	17.7 ± 1.1	5.7 ± 0.8^{g,h}	4.2 ± 0.4^{g,h}	0.3 ± 0.0^{g}	32.3 ± 0.8	16.2 ± 1.6^{g}	8.0 ± 0.8^{g}
Cholesterol	19.0 ± 0.4^{h}	1.7 ± 0.1^{g}	18.5 ± 1.9	7.3 ± 0.6^{g}	5.7 ± 0.7^{g}	1.1 ± 0.2^{h}	31.9 ± 0.3	11.9 ± 1.3^{h}	6.1 ± 0.7^{g}
Oxidized cholesterol	15.7 ± 0.4^{h}	0.5 ± 0.1^{h}	18.4 ± 1.4	4.3 ± 0.2^{h}	2.9 ± 0.4^{h}	0.3 ± 0.0^{g}	33.2 ± 0.5	16.6 ± 0.4^{g}	13.0 ± 1.9^{h}
ANOVA *f* value									
Age	$P < 0.01$	NS	NS	$P < 0.01$	NS	NS	NS	$P < 0.01$	NS
Sterol	$P < 0.01$	$P < 0.01$	NS	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Interaction	$P < 0.01$	NS	NS	NS	NS	NS	$P < 0.05$	NS	NS

[a]Data are presented as means ± SE for six rats in each group.
[b]Data were analyzed by two-way analysis of variance (ANOVA). When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test.
[c–e,g–i]Values in the same age group without a common superscript letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult age rats at $P < 0.05$. NS, not significant.

in young rats, but it was not the case for oxidized cholesterol. The composition of acidic steroids was also modified by dietary oxidized cholesterol, as characterized by the different changes in the ratios of primary to secondary bile acids, particularly in young rats.

Contents of oxidized cholesterols in serum and liver. Table 5 shows the contents of various oxidized cholesterol in serum and in liver. In both young and adult rats, dietary oxidized cholesterol elevated the level of each oxidized cholesterol in serum. The concentration of 7α-hydroxycholesterol, 5β-epoxycholesterol, and unknown steroids was higher in adult rats than in young rats. 5β- and 5α-Epoxycholesterols, cholestanetriol, and 7-ketocholesterol were detected only in rats fed oxidized cholesterol. 7α- and 7β-Hydroxycholesterols were detected even in rats that were not fed oxidized cholesterol. Similar results were observed in the liver.

TBA value of blood and liver. The blood TBA value was higher in the oxidized cholesterol groups than in the cholesterol-free groups for both ages, but particularly in adult rats (Fig. 4), whereas the value of the cholesterol groups was comparable to the cholesterol-free groups. In contrast, there was no increase in liver TBA value after feeding with oxidized cholesterol, while cholesterol significantly reduced the liver TBA value.

Effect of oxidized cholesterols on $CD4^+$ and $CD8^+$ splenic T-cell subsets. The percentage of $CD4^+$ and $CD8^+$ cells in splenic lymphocytes of rats fed different diets is summarized in Table 6. The proportion of $CD4^+$ increased, whereas that of $CD8^+$ decreased with age. As a result, the ratio of $CD4^+/CD8^+$ increased in adult rats. Although the results were not significant, dietary cholesterol tended to lower the ratio in adult rats, while oxidized cholesterol did not influence it.

DISCUSSION

This study was designed to elucidate how oxidized cholesterol influences the changes in lipid metabolism during aging. Care was taken to rule out the possible toxic effect of oxidized cholesterol, and it was given at a 0.2% level under the pair-feeding protocol. Despite pair-feeding, oxidized cholesterol reduced weight gain in young, but not in adult, rats. Kandutsch *et al.* (32) found that dietary 25-hydroxycholesterol or 7-ketocholesterol causes a loss of body weight in mature mice (eight-week-old), and both weight loss and suppression of growth in immature mice (four-week-old). Thus, oxidized cholesterol exerted a greater influence on growth parameters of young rats whose homeostatic ability is not fully matured. Furthermore, oxidized cholesterol did not increase liver

TABLE 4
Effect of Dietary Cholesterol and Oxidized Cholesterol on Fecal Acidic and Neutral Sterol Excretion in Young and Adult Rats[a]

	Young			Adult			ANOVA *f* value[b]		
	Cholesterol-free	Cholesterol	Oxidized cholesterol	Cholesterol-free	Cholesterol	Oxidized cholesterol	Age	Sterol	Interaction
Dried fecal weight (g/d)	1.65 ± 0.11*	1.62 ± 0.15*	1.56 ± 0.11*	2.16 ± 0.16	2.38 ± 0.15	2.38 ± 0.16	$P < 0.01$	NS	NS
Neutral sterol (mg/d)									
Coprostanol	0.23 ± 0.11^{e,*}	1.59 ± 0.34^{f,*}	3.10 ± 0.53^{g}	2.55 ± 0.57^{h}	5.53 ± 0.34^{i}	3.21 ± 0.18^{h}	$P < 0.01$	$P < 0.01$	$P < 0.01$
Cholesterol	1.94 ± 0.31^{e}	9.56 ± 2.51^{f}	2.79 ± 0.62^{g}	2.13 ± 0.34^{h}	8.85 ± 1.05^{i}	3.73 ± 0.24^{h}	NS	$P < 0.01$	NS
Unknown sterols[c]	3.86 ± 0.37^{e,*}	3.99 ± 0.84^{e}	9.37 ± 0.71^{f,*}	5.82 ± 0.70^{h}	5.88 ± 0.68^{h}	13.44 ± 0.34^{i}	$P < 0.01$	$P < 0.01$	NS
Total neutral sterol	5.06 ± 0.44^{e,*}	14.39 ± 3.01^{f}	14.29 ± 0.99^{g,*}	9.23 ± 1.43^{h}	20.18 ± 1.43^{i}	18.85 ± 0.38^{i}	$P < 0.01$	$P < 0.01$	NS
Acidic sterol									
Lithocholic	0.58 ± 0.08	0.67 ± 0.17	0.47 ± 0.06*	0.49 ± 0.15^{h,i}	0.36 ± 0.08^{h}	1.00 ± 0.24^{i}	NS	NS	$P < 0.05$
Deoxycholic	1.94 ± 0.27^{e,*}	3.46 ± 0.53^{f}	2.83 ± 0.20^{e,f,*}	5.61 ± 0.47	4.54 ± 1.32	5.02 ± 0.43	$P < 0.01$	NS	NS
Chenodeoxycholic	0.64 ± 0.12^{e}	1.34 ± 0.22^{f}	1.41 ± 0.09^{f}	0.45 ± 0.18	0.83 ± 0.13	0.56 ± 0.07	$P < 0.01$	$P < 0.01$	$P < 0.05$
Hyodeoxycholic + ursodeoxycholic	0.69 ± 0.03^{e,*}	1.32 ± 0.18^{f,*}	1.61 ± 0.10^{f,*}	4.46 ± 0.37^{h}	6.91 ± 0.31^{i}	3.12 ± 0.24^{j}	$P < 0.01$	$P < 0.01$	$P < 0.01$
Cholic	2.63 ± 0.22^{e}	2.57 ± 0.41^{e,*}	1.44 ± 0.17^{f}	2.02 ± 0.39^{h}	1.09 ± 0.05^{i}	1.38 ± 0.13^{h,i}	$P < 0.01$	$P < 0.01$	NS
α-Muricholic	1.35 ± 0.18^{e,*}	3.30 ± 0.39^{f,*}	1.22 ± 0.07^{e,*}	0.76 ± 0.15^{h}	0.90 ± 0.17^{h}	2.02 ± 0.08^{i}	$P < 0.01$	$P < 0.01$	$P < 0.01$
β-Muricholic	0.92 ± 0.14^{e}	2.51 ± 0.44^{f,*}	0.89 ± 0.09^{e,*}	0.88 ± 0.20^{h}	0.68 ± 0.27^{h}	1.88 ± 0.20^{i}	NS	$P < 0.05$	$P < 0.01$
Unknowns[d]	0.28 ± 0.06^{e}	0.56 ± 0.10^{f}	0.03 ± 0.02^{g,*}	0.42 ± 0.12^{h}	0.95 ± 0.25^{i}	0.99 ± 0.07^{i}	$P < 0.01$	NS	$P < 0.01$
Total acidic sterol	9.25 ± 0.53^{e,*}	16.28 ± 1.78^{f}	10.46 ± 0.53^{e,*}	16.07 ± 2.05	15.48 ± 2.31	17.36 ± 1.12	$P < 0.01$	NS	$P < 0.01$
Deoxycholic/cholic	0.79 ± 0.16^{e,*}	1.58 ± 0.33^{e,f,*}	2.10 ± 0.29^{f,*}	3.23 ± 0.75	4.42 ± 1.07	3.77 ± 0.48	$P < 0.01$	NS	NS
Lithocholic/chenodeoxycholic	0.98 ± 0.14^{e}	0.50 ± 0.09^{f}	0.33 ± 0.05^{f,*}	1.28 ± 0.26^{h,i}	0.47 ± 0.10^{h}	1.93 ± 0.51^{i}	$P < 0.05$	NS	NS
Total sterols (mg/d)	15.3 ± 0.8^{e,*}	31.3 ± 4.6^{f}	25.7 ± 1.4^{f,*}	28.6 ± 3.7	36.2 ± 2.1	36.8 ± 1.7	$P < 0.01$	NS	$P < 0.01$

[a]Data are presented as means ± SE for six rats in each group.
[b]Data were analyzed by two-way ANOVA. When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test. Abbreviations as in Table 3.
[c]Composed of more than 20 components including oxidized cholesterols and plant sterols and their metabolites, and [d]more than five unknown components.
[e–g,e–i]Values in the same age group without a common superscript letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult age rats at $P < 0.05$.

TABLE 5
Effect of Dietary Cholesterol and Oxidized Cholesterol on Sterol Composition of Serum and Liver in Young and Adult Rats[a]

	Young			Adult			ANOVA *f* value[b]		
Component	Cholesterol-free	Cholesterol	Oxidized cholesterol	Cholesterol-free	Cholesterol	Oxidized cholesterol	Age	Sterol	Interaction
Serum				(mg/dL)					
Cholesterol	84.3 ± 15.1^{e,*}	174 ± 18.4^{f}	96.1 ± 7.7^{e,*}	134 ± 6.4	122 ± 19.4	129 ± 6.5	NS	$P < 0.05$	$P < 0.01$
7α-Hydroxycholesterol	0.14 ± 0.07^{e}	0.26 ± 0.12^{e}	0.99 ± 0.10^{f,*}	0.05 ± 0.03^{h}	0.09 ± 0.06^{h}	1.50 ± 0.21^{i}	NS	$P < 0.01$	NS
7β-Hydroxycholesterol	0.69 ± 0.03^{e,*}	1.26 ± 0.08^{f,*}	0.99 ± 0.13^{f}	1.44 ± 0.09^{h}	0.91 ± 0.07^{i}	0.93 ± 0.07^{i}	NS	NS	$P < 0.01$
5β-Epoxycholesterol	n.d.	n.d.	1.26 ± 0.14*	n.d.	n.d.	2.96 ± 0.13	$P < 0.01$	$P < 0.01$	$P < 0.01$
5α-Epoxycholesterol	n.d.	n.d.	0.37 ± 0.05	n.d.	n.d.	0.36 ± 0.01	NS	$P < 0.01$	NS
Cholestanetriol	n.d.	n.d.	1.11 ± 0.11*	n.d.	n.d.	0.33 ± 0.02	$P < 0.01$	$P < 0.01$	$P < 0.01$
7-Ketocholesterol	n.d.	n.d.	0.47 ± 0.04	n.d.	n.d.	0.43 ± 0.04	NS	$P < 0.01$	NS
Unknown sterols[c]	0.83 ± 0.10*	1.01 ± 0.08	1.09 ± 0.14*	1.54 ± 0.14^{h}	1.16 ± 0.04^{i}	3.29 ± 0.13^{j}	$P < 0.01$	$P < 0.01$	$P < 0.05$
Liver				(μg/g)					
Cholesterol	5562 ± 542^{e}	27519 ± 1469^{f}	2982 ± 351^{e,*}	5830 ± 753^{h}	24468 ± 2403^{i}	7320 ± 1602^{h}	NS	$P < 0.01$	$P < 0.05$
7α-Hydroxycholesterol	n.d.*	n.d.*	42.1 ± 10.0*	4.0 ± 2.5	4.0 ± 4.0	13.1 ± 7.2	NS	$P < 0.01$	$P < 0.01$
7β-Hydroxycholesterol	52.8 ± 3.3^{e,*}	78.1 ± 4.7^{f,*}	50.5 ± 5.6^{e}	77.2 ± 8.7^{h}	145 ± 12.0^{i}	71.6 ± 15.5^{h}	$P < 0.01$	$P < 0.01$	$P < 0.05$
5β-Epoxycholesterol	n.d.	n.d.	53.4 ± 8.8*	n.d.	n.d.	171 ± 34	$P < 0.01$	$P < 0.01$	$P < 0.01$
5α-Epoxycholesterol	n.d.	n.d.	19.8 ± 1.4*	n.d.	n.d.	64.7 ± 16.2	$P < 0.01$	$P < 0.01$	$P < 0.01$
Cholestanetriol	n.d.	n.d.	16.7 ± 1.4	n.d.	n.d.	11.3 ± 2.7	NS	$P < 0.01$	NS
7-Ketocholesterol	n.d.	n.d.	5.8 ± 0.9*	n.d.	n.d.	1.5 ± 1.0	$P < 0.01$	$P < 0.01$	$P < 0.01$
Unknown sterols[c]	27.5 ± 2.2^{e,*}	54.9 ± 3.0^{f,*}	79.0 ± 8.3^{g}	63.3 ± 7.7^{h}	91.4 ± 4.5^{h,i}	124 ± 28^{i}	$P < 0.01$	$P < 0.01$	NS

[a]Data are presented as means ± SE for six rats in each group.
[b]Data were analyzed by two-way ANOVA. When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test. Abbreviations as in Table 3.
[c]Composed of more than 20 components. Mainly plant sterols in the noncholesterol and cholesterol-added groups, whereas oxidized cholesterols in addition to plant sterols in the oxidized cholesterol group.
[e–i]Values in the same age group without a common superscript letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult age rats at $P < 0.05$. n.d., Not detected.

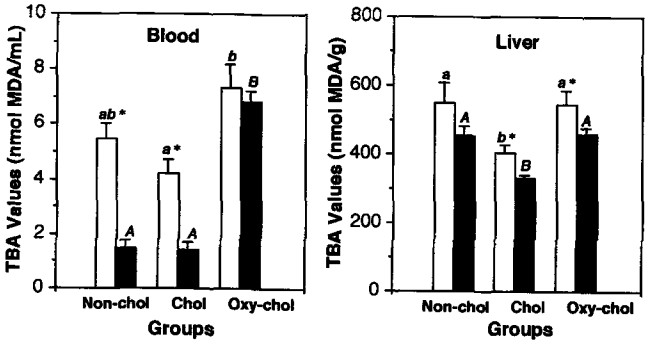


FIG. 4. Effect of dietary cholesterol and oxidized cholesterol on blood and liver thiobarbituric acid (TBA) values in young and adult rats. Data are presented as means ± SE for six rats in each group. □, Young; ■, adult. Data were analyzed by two-way analysis of variance. When the *f* value for age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test. ^{a,b,A,B}Values without a common superscript letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult rats at $P < 0.05$. Abbreviations as in Figure 2. The following significant difference was observed: blood TBA, A, S and A × S; liver TBA, A and S. MDA, malondialdehyde.

weight, in contrast to cholesterol, and slightly increased the serum GPT level in adult, but not in young, rats (cholesterol-free, 19.2 ± 1.6; cholesterol, 20.9 ± 0.8; oxidized cholesterol, 23.9 ± 1.2 IU/L; the difference between two extremes was significant, $P < 0.05$). 7-Ketocholesterol at a dietary level of 0.5% appears to have toxic effects as indicated by the reduction of body and liver weights after 66 h feeding (33). Therefore, oxidized cholesterol exhibits potent toxicity toward hepatocytes and could impair liver function, particularly in adults. Oxidized cholesterol shows strong cytotoxicity (3) and inhibition of cell growth (34) *in vitro*.

TABLE 6
Effect of Dietary Cholesterol and Oxidized Cholesterol on CD4⁺ and CD8⁺ T-Cells in Splenic Lymphocyte Subsets in Young and Adult Rats[a]

Groups	CD4⁺ (%)	CD8⁺ (%)	CD4⁺/CD8⁺
Young			
Cholesterol-free	49.0 ± 1.4*	33.0 ± 1.6*	1.5 ± 0.1*
Cholesterol	47.8 ± 1.3*	32.9 ± 1.7*	1.5 ± 0.1*
Oxidized cholesterol	47.5 ± 2.9*	30.2 ± 1.0*	1.6 ± 0.1*
Adult			
Cholesterol-free	61.2 ± 0.6^{c,d}	21.2 ± 2.2	3.1 ± 0.3
Cholesterol	60.2 ± 0.4^{c}	22.9 ± 1.9	2.7 ± 0.2
Oxidized cholesterol	63.3 ± 1.3^{d}	19.9 ± 1.3	3.2 ± 0.2
ANOVA *f* value[b]			
Age	$P < 0.01$	$P < 0.01$	$P < 0.01$
Sterol	NS	NS	NS
Interaction	NS	NS	NS

[a]Data are presented as means ± SE for six rats in each group.
[b]Data were analyzed by two-way ANOVA. When the *f* value for the age or sterol effect was significant ($P < 0.05$), the differences between means were inspected by Duncan's multiple range test. Abbreviations as in Table 3.
^{c,d}Values in the same age group without a common superscript letter are significantly different at $P < 0.05$. *Significantly different from the corresponding value in adult age rats at $P < 0.05$.

The activity of hepatic HMG–CoA reductase was lower in adult rats than in young rats, as reported previously (35,36). The depressive effect on HMG–CoA reductase activity was more marked in the rats fed oxidized cholesterol, especially in adults under the conditions of these experiments. Various oxidized cholesterols have been shown to be potent inhibitors of sterol biosynthesis *in vitro* (5). Similarly, 7-ketocholesterol or 24(S),25-epoxycholesterol lowered HMG–CoA reductase activity after short-term feeding (33,37), although Kandutsch *et al.* (32) reported that dietary 25-hydroxycholesterol or 7-ketocholesterol did not depress hepatic cholesterol synthesis. However, the decrease of HMG–CoA reductase activity by oxidized cholesterol as observed in these studies could be modified by the development of tolerance to oxidized cholesterol when it is fed for a relatively long time. A possible explanation of these diverse observations is the report of Vargas *et al.* (38) that the oxidized cholesterol mixture is a potent inhibitor of hepatic HMG–CoA reductase after 14 days of feeding. Therefore, oxidized cholesterol may accelerate the impairment of hepatic cholesterol synthesis associated with aging. The depression of cholesterol synthesis could, in turn, lead to the reduction of the liver cholesterol level, particularly in young rats, in spite of the different response of the reductase activity as observed in the present study. Young animals fed oxidized cholesterol also tended to have lower concentrations of esterified cholesterol in liver compared to those fed a cholesterol-free diet. The amount of cholesterol ester in livers of rats fed cholesterol was also significantly higher than those fed a cholesterol-free diet (cholesterol-free, 3.38 ± 0.49; cholesterol, 23.8 ± 1.6; oxidized cholesterol, 1.38 ± 0.28 mg/g liver; the difference between cholesterol and other two groups was significant, $P < 0.05$). Oxidized cholesterol may cause the reduction of liver acyl-CoA:cholesterol *O*-acyltransferase (ACAT) activity in young but not in adult rats, although Smith and Johnson (39) reported a stimulation of ACAT activity by oxidized cholesterols. It is also known that short-term 7-ketocholesterol feeding increases hepatic microsomal esterified cholesterol content (33). Thus, oxidized cholesterol may disturb cholesterol homeostasis, such as biosynthesis and esterification, in ways that contribute to the aging of tissues. Elucidation of the effect of oxidized cholesterol on ACAT and cholesterol ester hydrolase *in vivo* deserves further study.

Choi *et al.* (35) found that the activity of cholesterol 7α-hydroxylase was low in adult rats as compared to young rats. It was also found that the excretion of fecal acidic steroids changes in the course of the aging process in some species (40,41). These findings are consistent with the present results, and reduction in activity of cholesterol 7α-hydroxylase by feeding of oxidized cholesterol in both age groups. The hydroxylase activity has been reported to be inhibited by various oxidized cholesterol *in vivo* (42). Thus, oxidized cholesterol may worsen the reduction of cholesterol 7α-hydroxylase activity with aging, and disturb cholesterol catabolism. The change in the composition of fecal acidic steroids at least partly indicates the differential effects of oxidized cholesterol on enzymes involved in bile acid conversion as compared to cholesterol. In

addition, oxidized cholesterol may interfere with the microbial conversion of bile acids in the large intestine.

On the other hand, the activity of hepatic Δ6 desaturase tended to be higher in the rats fed oxidized cholesterol than in those fed cholesterol, as reported previously (12). This difference was significant in young rats, although it is well known that dietary cholesterol decreases the desaturase activity (43). Consequently, the desaturation index [(20:3n-6 + 20:4n-6)/18:2n-6] significantly increased in liver phospholipids of rats fed oxidized cholesterol compared to cholesterol both in young and adult rats. Therefore, oxidized cholesterol may alter the production of eicosanoids, as the supply of free substrate is one determinant of eicosanoid production (44,45).

The TBA values of blood were higher in rats fed oxidized cholesterol than in the other groups of rats of both ages, but this was not the case in the liver. It is, however, at least plausible that peroxidized cholesterol may promote peroxidation of fatty acids.

Dietary oxidized cholesterol influenced the concentration of triglyceride and phospholipid in the liver and serum, especially in the liver of adult rats. The same tendency has been observed in plasma, but not in liver, of the chicks fed oxidized cholesterol (14). Although it is understood that hepatic triglyceride concentration increases with age (17), it significantly decreased when rats were fed oxidized cholesterol. This may be one specific effect of oxidized cholesterol.

T-cells were characterized by the cell surface markers CD4 and CD8, which bind to nonpolymorphic regions of MHC classes II and I, respectively (46). $CD4^+$ T-cells act as helper/inducers and $CD8^+$ T-cells as cytotoxic/suppressors. The percentage of $CD4^+$ cells in spleen lymphocytes increased and that of $CD8^+$ cells decreased with age, suggesting the promotion of immunoglobulin production. However, as oxidized cholesterol compared to cholesterol did not lower the $CD4^+/CD8^+$ ratio in adult rats, it seems unlikely that oxidized cholesterol influences the depression of immunoglobulin production found with age (in contrast to cholesterol). More detailed studies on this observation are required.

In conclusion, the present study demonstrated diverse undesirable effects of dietary oxidized cholesterol on growth and lipid metabolism in young and adult rats. In contrast to cholesterol, oxidized cholesterol did not modify the splenic T-lymphocyte subsets. Therefore, dietary oxidized cholesterol appears to worsen various parameters of homeostasis, irrespective of the age of the animals. In this context, studies are required to elucidate the gender-dependence of the effects of oxidized cholesterol, as there are considerable differences in lipid metabolism between male and female rats. An attempt to study the effects of oxidized cholesterol over a greater portion of natural life span would also be valuable

ACKNOWLEDGMENT

This study was supported by a grant from the Research Projects on Health and Aging, Ministry of Public Welfare, Japan (90A1102).

REFERENCES

1. Kubows, S. (1993) *Nutrition Reviews 51,* 33–49.
2. Morin, R.J., Hu, B., Peng, S.-K., and Sevanian, A. (1991) *J. Clin. Lab. Anal. 5,* 219–225.
3. Baranowski, A., Adams, C.W.M., Bayliss High, O.B., and Bowyer, D.B. (1982) *Atherosclerosis 41,* 255–266.
4. Sevanian, A., and Peterson, A.R. (1986) *Food Chem. Toxic. 24,* 1103–1110.
5. Erickson, S.K., Shrewsbury, M.A., Gould, R.G., and Cooper, A.D. (1980) *Biochim. Biophys. Acta 620,* 70–79.
6. Nourooz-Zadeh, J., and Appelqvist, L.-A. (1987) *J. Food Sci. 52,* 57–62.
7. Nourooz-Zadeh, J., and Appelqvist, L.-A. (1988) *J. Food Sci. 53,* 74–79.
8. Highly, N.A., and Taylar, S.L. (1986) *Meat Sci. 16,* 175–188.
9. Ohshima, T., Li, N., and Koizumi, C. (1993) *J. Am. Oil Chem. Soc. 70,* 595–600.
10. Osada, K., Kodama, T., Cui, L., Yamada, K., and Sugano, M. (1993) *J. Agric. Food Chem. 41,* 1893–1898.
11. Osada, K., Sasaki, E., and Sugano, M. (1994) *Lipids 29,* 555–559.
12. Osada, K., Kodama, T., Cui, L., Ito, Y., and Sugano, M. (1994) *Biosci. Biotech. Biochem. 58,* 1062–1069.
13. Emanuel, H.A., Hassel, C.A., Addis, P.B., Bergmann, S.D., and Zavoral, J.H. (1991) *J. Food Sci. 56,* 843–847.
14. Cho, B.H.S., Egwin, O.O., and Fahey, Jr., G.C. (1986) *Comp. Biochem. Physiol. 83B,* 767–770.
15. Halliwell, B. (1991) *Free Radicals in Health and Disease 87,* 576–584.
16. Choi, Y.-S., Sugano, M., and Ide, T. (1988) *Mech. Ageing Develop. 44,* 91–99.
17. Choi, Y.-S., Ide, T., and Sugano, M. (1987) *Exp. Gerontol. 22,* 339–349.
18. Hobbs, M.V., Weigle, W.O., Noonan, D.J., Torbett, B.E., McEvilly, R.J., Koch, R.J., Cardenas, G.J., and Ernst, D.N. (1993) *J. Immunol. 150,* 3602–3614.
19. American Institute of Nutrition (1977) *J. Nutr. 107,* 1340–1348.
20. Shapiro, D.J., Nordstorm, J.L., Mitschelen, J.J., Rodwell, V.W., and Schmike, R.T. (1974) *Biochim. Biophys. Acta 370,* 171–178.
21. Van Cantfort, J., Renson, J., and Gielen, J. (1975) *Eur. J. Biochem. 55,* 22–31.
22. Svensson, L. (1983) *Lipids 18,* 171–178.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
24. Ide, T., Okamatsu, H., and Sugano, M. (1978) *J. Nutr. 108,* 601–612.
25. Ikeda, I., Tomari, Y., and Sugano, M. (1989) *J. Nutr. 119,* 1383–1387.
26. Sugano, M., Yamada, Y., Yoshida, K., Hashimoto, Y., Matsuo, T., and Kimoto, M. (1988) *Atherosclerosis 72,* 115–122.
27. Yagi, K. (1976) *Biochem. Med. 15,* 212–216.
28. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochem. 95,* 351–358.
29. Fisher, R.A. (1970) *Statistical Methods for Research Workers,* 14th edn., pp. 140–142, Oliver and Boyd, Edinburgh, Scotland.
30. Duncan, D.B. (1955) *Biometrics 11,* 1–42.
31. Gomez, K.A., and Gomez, A.A. (1984) *Statistical Procedures for Agricultural Research,* 2nd edn., pp. 91–97, John Wiley & Sons, New York.
32. Kandutsch, A.A., Heiniger, H-J., and Chen, H.W. (1977) *Biochim. Biophys. Acta 486,* 260–272.
33. Erickson, S.K., Cooper, A.D., Matsui, S.M., and Gould, R.G. (1977) *J. Biol. Chem. 252,* 5186–5193.
34. Chen, H.W., Kandutsch, A.A., and Waymouth, C. (1974) *Nature 251,* 419–421.

35. Choi, Y.-S., Tomari, Y., Sugano, M., and Ide, T. (1987) *Mech. Ageing Dev. 41,* 149–159.
36. Takeuchi, N., Kukita, H., Katayama, Y., Koga, M., and Uchida, K. (1980) *Exp. Gerontol. 15,* 15–23.
37. Saucier, S.E., Kandutsch, A.A., Clark, D.S., and Spencer, T.A. (1993) *Biochim. Biophys. Acta 1166,* 115–123.
38. Vargas, R.E., Allard, J.B., Biggert, M.D., and Naber, E.C. (1986) *Poultry Sci. 65,* 1333–1342.
39. Smith, L.L., and Johnson, B.H. (1989) *Free Radical Biol. Med. 7,* 285–332.
40. Hruza, Z., and Zbuzkova, V. (1973) *Exp. Gerontol. 8,* 29–37.
41. Uchida, K., Nomura, Y., Kadowaki, M., Takase, H., Takano, K., and Takeuchi, N. (1978) *J. Lipid Res. 19,* 544–552.
42. Schwartz, M.A., and Margolis, S. (1983) *J. Lipid Res. 24,* 28–33.
43. Grag, M.L., Snoswell, A.M., and Sabine, J.R. (1986) *Prog. Lipid Res. 25,* 639–644.
44. Hashimoto, A., Katagiri, M., Torii, S., Dainaka, J., Ichikawa, A., and Okuyama, H. (1988) *Prostaglandins 36,* 3–16.
45. Hammarström, S. (1983) *Ann. Rev. Biochem. 52,* 355–377.
46. Cantor, H., and Boyse, E.A. (1975) *J. Exp. Med. 141,* 1376–1399.

[Received September 12, 1994; and in revised form February 10, 1995; Revision accepted February 24, 1995]

Dietary Fats Rich in Saturated Fatty Acids (12:0, 14:0, and 16:0) Enhance Gallstone Formation Relative to Monounsaturated Fat (18:1) in Cholesterol-Fed Hamsters

Satya S. Jonnalagadda, Elke A. Trautwein[1] and K.C. Hayes*

Foster Biomedical Research Laboratory, Brandeis University, Waltham, Massachusetts 02254

ABSTRACT: To test the possibility that dietary palmitic acid (16:0) may be lithogenic, different fats were blended to exchange 18:1 in olive oil with either 16:0 in palm stearin, 12:0 + 14:0 in coconut oil, or 14:0 + 16:0 in butterfat. Dietary 18:2 was held constant at 1.2% energy (en) (with extra safflower oil as needed) in these four purified diets containing low fat (11% of total energy) and 0.4% cholesterol. A fifth, high-fat diet provided 40% of the total energy as the 16:0-rich blend. All hamsters fed the low-fat, 16:0-rich blend for six weeks developed cholesterol gallstones (8/8). Although the gallstone incidence was lower for the 12:0 + 14:0-rich diet (5/8), the severity of stone formation in affected hamsters was equal to that in the low-fat, 16:0-rich group. Mucin accumulation in gallbladder bile was often associated with cholesterol gallstones in diets containing 16:0, but was minimal in 18:1-rich and 12:0 + 14:0-rich groups. Neither the lithogenic index (all >1.0), plasma lipids, nor liver cholesterol was a selective predictor of stone formation. The high-fat, 16:0-rich diet actually decreased cholesterol stone incidence (3/8) and severity, but yielded a high incidence of pigment stones (5/8). Thus, saturated fat and 16:0 *per se* were not responsible for the exaggerated lithogenesis. Because the antilithogenic 18:1-rich diet also normalized the 18:2 intake (1.2% en) relative to previous butter diets (0.3% en), the potential importance of essential fatty acids (EFA) deficiency in the model was tested in a second study by feeding graded amounts of 18:2 (0.3, 0.6, 0.9, and 1.2% en) as safflower oil in four low-fat, butter-rich diets (11% en as fat) without alleviating gallstone incidence or severity. These studies indicate that substitution of 18:1 for saturated fatty acids in low-fat diets reduces gallstone formation without affecting the lithogenic index. Furthermore, intake of 18:2 at or below the EFA requirement does not appear to be a major factor in this model.

Lipids 30, 415–424 (1995).

[1]Present address: Institute of Human Nutrition and Food Science, University of Kiel, 24105 Kiel, Germany.

*To whom correspondence should be addressed.

Abbreviations: ACAT, acyl cholesterol acyl transferase; ANOVA, analysis of variance; apo B, apolipoprotein B; apo E, apolipoprotein E; CE, cholesteryl ester; EFA, essential fatty acid; en, energy; HDL, high-density lipoprotein; HI, hydrophobicity index; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; LDLr, low density lipoprotein receptor; LI, lithogenic index; PUFA, polyunsaturated fatty acids; TC, total cholesterol.

The roles of dietary cholesterol and the type of dietary fat as etiologic agents for cholesterol gallstones have received considerable attention but remain unresolved (1). Most successful models of cholelithiasis depend on the feeding of excessive cholesterol, but the type of fat also influences bile cholesterol concentration and bile acid composition (2–4). The impact of fat saturation on gallstone development in any species is not clear, although one study in men suggested that polyunsaturated fatty acid (PUFA) increased the excretion of biliary cholesterol while decreasing the mole percent of bile acids (5). On the other hand, epidemiological evidence suggests that consumption of PUFA may protect against gallstones in women (6).

Purified diets containing butterfat (4–5%) and 0.3–0.4% cholesterol are especially lithogenic in hamsters (7–9). Substitution of olive oil for butterfat prevented stone formation completely, whereas palmitic acid added to the diet enhanced cholesterol gallstone formation (8). However, previous studies that compared dietary fats during gallstone induction have not always controlled for multiple changes in individual dietary fatty acids, even though evidence indicated that 16:0 might be most lithogenic.

In the present study, all fatty acids were controlled (equalized between diets) in order to test whether substitution of a dietary monounsaturated fatty acid (18:1) for specific saturated fatty acids (12:0, 14:0, 16:0) in coconut oil, butterfat, and palm stearin would alter the incidence of cholesterol cholelithiasis in the hamster. The dietary 18:2 (from safflower oil) was kept constant in all four diets at 1.2% energy (en). In addition, the effect of a high-16:0 intake was examined to assess the quantitative importance of that specific saturated fatty acid on gallstones.

In a second investigation, graded amounts of 18:2 (0.3, 0.6, 0.9, and 1.2% en) were added to the low-fat, butter diet (11% en) to reexamine the possible protective influence of PUFA originally noted in the essential fatty acid (EFA)-deficient model of Dam (10).

MATERIALS AND METHODS

Animals and diets. Male golden Syrian hamsters (*Mesocricetus auratus*, Lakeview strain), approximately 20 days old,

Copyright © 1995 by AOCS Press

weighing 50–60 g were obtained from Charles River Laboratories, (Kingston, NY). The animals were randomly assigned to five groups (in Experiment 1, n = 8; Experiment 2, n = 12) and fed purified diets for six weeks. The purified diets were made by blending different fats to exchange olive oil (18:1-rich) with either palm stearin (16:0-rich), coconut oil (12:0 + 14:0-rich), or butterfat (14:0 + 16:0-rich). Dietary 18:2 was kept constant at 1.2% en (with extra safflower oil as needed) in these purified diets, in which fat supplied 11% of the total calories; and cholesterol was added at 0.4%. A fifth diet contained the 16:0-rich blend at 40% total energy (Tables 1 and 2). In the second experiment graded amounts of 18:2-rich safflower oil (0.3, 0.6, 0.9, and 1.2% en) were blended into the low-fat, gallstone-inducing butter diet (11% en) (Table 3) to examine the impact of the transition from a subnormal to adequate intake of EFAs.

The hamsters were housed in groups of four per cage and kept in a temperature-controlled environment with a 12-h light–dark cycle (lights on 1800 h). The diets were fed *ad libitum* (25 g wet weight per hamster daily), and the daily food intake was monitored. Water was provided *ad libitum*. Body weights were monitored on a weekly basis. All protocols and procedures were approved by the Brandeis University Animal Care and Use Committee.

Plasma lipid analysis. Blood samples were drawn from animals fasted overnight (18 h), at baseline, and at the end of six weeks. Blood samples were collected under light anesthesia, into an EDTA-wetted syringe by cardiac puncture, and plasma was separated by centrifugation at 12,000 × *g* for 15 min. Plasma total cholesterol (TC), high-density lipoprotein (HDL)-cholesterol (HDL-C), and triglycerides were determined by enzymatic assays (Sigma kit 352 for cholesterol and 336 for triglycerides; Sigma Chemical, St. Louis, MO). HDL-cholesterol was assayed following Mg^{2+}-phosphotungstate precipitation of lipoproteins containing apolipoprotein B (apo B) and apo E using the HDL-cholesterol Reagent Set® (Boehringer Diagnostics, Indianapolis, IN), according to the procedure described by Weingard and Daggy (11).

Necropsy and gallstone evaluation. After six weeks, hamsters were exsanguinated under anesthesia, and the liver, cecum, and small intestine were excised, blotted, and weighed. A portion of the liver was taken and frozen for hepatic cholesterol analysis. Gallbladder bile was aspirated, weighed, and analyzed for biliary lipids and bile acids. The gallbladder was dissected from the liver, opened under a dissecting microscope, and examined for gallstones under regular and polarized light microscopy (12).

A gallstone score (0–10) was generated by one of us (KCH, blinded) based on gradations in gallstone formation as well as number and size of stones. Low scores (for cholesterol stones) were represented by a cloudy mucin gel with cholesterol crystals (sludge), whereas the highest score was characterized by a gallbladder distended with numerous, 0.5–2.0 mm rounded almost pure-white stones that essentially displaced bile. Parallel scores for pigment stones ranged from a greenish, inspissated gel to numerous large, well-formed, pleomorphic green-brown to black stones, again completely filling the gallbladder. Occasionally distinct mixtures of the two types are observed, and occasionally cholesterol is deposited around a pigmented nucleus. Pigment stones often

TABLE 1
Composition of the Purified Diets Containing 0.4% Cholesterol and Different Dietary Fatty Acids (Experiment 1)[a]

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
		(g/100 g)			
Casein	20	20	20	20	20
Cornstarch	33.5	33.5	33.5	33.5	18.5
Glucose	20	20	20	20	20
Cellulose	10	10	10	10	10
Wheat bran	5	5	5	5	5
Fat					
Butter[b]	0.4	—	4.4	—	—
Safflower oil	—	0.6	0.6	0.4	1.5
Olive oil	3.4	—	—	—	—
Coconut oil	—	4.4	—	—	—
Palm stearin	—	—	—	4.6	18.5
Beef tallow[b]	1.2	—	—	—	—
Vitamin mix[c]	1.2	1.2	1.2	1.2	1.2
Mineral mix[c]	4.6	4.6	4.6	4.6	4.6
Choline-Cl_3	0.3	0.3	0.3	0.3	0.3
Cholesterol	0.4	0.4	0.4	0.4	0.4

[a]Diets were fed as starch gels, prepared by withholding 6 g cornstarch per 100 g mix from the formulation and premixing it with 80 mL of simmering water to form a gel to which the remaining ingredients were added.
[b]Cholesterol-stripped (Source Food Technology Corp., Burnsville, MN).
[c]The composition of the Hayes-Cathcart vitamin mix and Ausman-Hayes mineral mix was detailed previously (Ref. 12).

TABLE 2
Fatty Acid Composition of the Fat Blends Fed in Experiment 1

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
	(% total fatty acids)				
12:0	1.0 (0.1)[a]	54.6 (6.1)	7.7 (0.8)	0.1 (0.0)	(0.0)
14:0	2.1 (0.2)	17.0 (1.9)	12.8 (1.4)	1.0 (0.1)	(0.4)
16:0	22.2 (2.5)	8.9 (1.0)	33.3 (3.7)	60.8 (6.7)	(26.8)
16:1	1.9 (0.2)	0.0 (0.0)	1.5 (0.2)	0.1 (0.0)	(0.0)
18:0	5.0 (0.6)	2.1 (0.2)	8.4 (0.9)	3.3 (0.4)	(1.6)
18:1	55.4 (6.1)	6.7 (0.7)	21.6 (2.4)	24.1 (2.7)	(10.8)
18:2	10.8 (1.2)	10.6 (1.2)	10.5 (1.2)	10.2 (1.1)	(4.4)
18:3	0.3 (0.0)	0.0 (0.0)	0.2 (0.0)	0.2 (0.0)	(0.0)

[a]Values in parentheses represent percent energy contributed by individual fatty acid.

TABLE 3
Composition of the Purified Diets Containing 5% Fat (butter-rich) and 0.4% Cholesterol and Graded Amounts of Linoleic Acid (safflower oil) (Experiment 2)[a]

	Dietary 18:2 percent energy (% en)			
	0.3% en	0.6% en	0.9% en	1.2% en
	g/100 g			
Casein	20	20	20	20
Cornstarch	33.5	33.5	33.5	33.5
Glucose	20	20	20	20
Cellulose	10	10	10	10
Wheatbran	5	5	5	5
Fat				
Butter	5	4.8	4.5	4.3
Safflower oil	—	0.2	0.5	0.7
Vitamin mix[b]	1.2	1.2	1.2	1.2
Mineral mix[b]	4.6	4.6	4.6	4.6
Choline Cl_3	0.3	0.3	0.3	0.3
Cholesterol	0.4	0.4	0.4	0.4

[a]Diets were fed as starch gels, prepared by withholding 6 g cornstarch per 100 g mix from the formulation and premixing it with 80 mL of simmering water to form a gel to which the remaining ingredients were added.
[b]The composition of the Hayes-Cathcart vitamin mix and Ausman-Hayes mineral mix was detailed previously (Ref. 12).

form in hamster bile (especially when fed purified diets) that is not supersaturated with cholesterol, whereas cholesterol stones never do.

Bile analysis and bile acid pool size. Gallbladder bile lipids were isolated using a modified Folch extraction (13). A 10 μL aliquot of gallbladder bile was extracted with 3 mL chloroform/methanol (2:1, vol/vol) and 0.75 mL of 1.12% KCl solution. Biliary cholesterol, in an aliquot of the evaporated chloroform layer redissolved in isopropanol, was measured using the enzymatic Wako free cholesterol C kit (Wako Chemicals, Richmond, VA). Biliary phospholipid was measured using the Wako phospholipids B kit. Biliary bile acid concentration was determined as conjugated bile acids using an isocratic high-performance liquid chromatography (HPLC) procedure adapted from Rossi *et al.* (14), as described previously (15).

Bile acid pool size. This was determined as the sum of bile acids in gallbladder bile and small intestine. Small intestines were homogenized completely (Tissuemizer; Tekmar, Cinncinati, OH), and 100 mg of homogenate was ground with anhydrous sodium sulfate prior to extraction with 6 mL of chloroform/methanol (2:1, vol/vol) and 1.5 mL of 1.12% KCl solution. An aliquot from the methanol phase was then assayed for bile acids. Total gallbladder bile acids was determined as the biliary bile acid concentration times the volume of bile collected from the gallbladder.

The lithogenic index (LI) was calculated based on the relative molar ratios of lipid components and total lipid concentration using a computerized version of cholesterol solubility (16). The hydrophobicity index (HI) for individual bile samples was determined using the procedure of Heuman (17).

Hepatic cholesterol analysis. Hepatic cholesterol was extracted by grinding a 100 mg portion of liver with anhydrous sodium sulfate and by extracting three times with chloroform/methanol (2:1, vol/vol). The liquid phase was evaporated to dryness and redissolved in chloroform. An aliquot was evaporated and redissolved in isopropanol for analysis of free cholesterol using HPLC (18). TC was determined enzymatically using the Sigma total cholesterol kit. Esterified cholesterol was calculated as the difference between the TC and free cholesterol.

Fecal bile acid and cholesterol excretion. In the first study, feces were collected over a three-day period from all hamsters. Fecal bile acids were analyzed by using a modified method of Suckling *et al.* (19). The feces were oven-dried overnight and pulverized before hydrolysis. A 0.1 g fecal sample was hydrolyzed in 10 M NaOH at 110°C overnight. An aliquot of the hydrolysate was evaporated and extracted with 3 mL of chloroform/methanol (2:1, vol/vol) and 0.75 mL of 1.12% KCl. An aliquot from the methanol/KCl layer was evaporated under nitrogen and redissolved in a mixture of calf serum/saline (1:1, vol/vol), and bile acid concentration was determined using the enzymatic Sigma bile acid kit (Kit 450-A). Cholesterol was determined in an aliquot of the chloroform layer using the Sigma TC kit.

Statistical analysis. Statistical differences for the low-fat diet comparisons in both Experiments 1 and 2 were calculated using a one-way analysis of variance (ANOVA) followed by Scheffe's F-test utilizing the SuperANOVA statistical software package (Abacus Concepts, Inc., Berkeley, CA). The difference between the low-fat and high-fat 16:0-rich diets (Experiment 1) was calculated using a Student's *t*-test (Statview 512$^+$; Brain Power Inc., Calabasas, CA).

RESULTS

Experiment 1

Plasma cholesterol and triglycerides. After six weeks of feeding purified diets rich in specific saturated or monounsaturated fatty acids in the presence of a high cholesterol intake, body and organ weights were normal and equal (Table 4), but the baseline plasma TC (150 ± 40 mg/dL) had more than doubled (Table 5). However, no significant differences were observed between low-fat treatment groups or between the low-

TABLE 4
Body and Organ Weights of Hamsters Fed a Purified Diet Containing 0.4% Cholesterol and Different Dietary Fatty Acids for Six Weeks (Experiment 1)[a]

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
Body weight (g)					
Initial	61 ± 3[b]	56 ± 4	55 ± 3[b]	59 ± 3	57 ± 3
Final	132 ± 8	129 ± 14	137 ± 8	132 ± 8	134 ± 9
Liver (g)	7.2 ± 0.4	6.6 ± 0.4	7.0 ± 0.9	6.9 ± 0.7	8.7 ± 0.4
Cecum (g)	2.1 ± 0.5	1.8 ± 0.4	2.0 ± 0.7	1.6 ± 0.2	1.6 ± 0.3
Small intestine (g)	1.9 ± 0.1	1.9 ± 0.2	2.1 ± 0.3	1.9 ± 0.1	2.3 ± 0.2

[a]Values are mean ± SD (n = 8).
[b]Means in a row sharing a common superscript are significantly different ($P < 0.05$) using analysis of variance and Scheffe's F-test.

TABLE 5
Plasma and Hepatic Lipid Concentrations in Hamsters Fed Purified Diets Containing 0.4% Cholesterol and Different Dietary Fatty Acids for Six Weeks (Experiment 1)[a]

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
			(mg/dL)		
Plasma lipids					
Total cholesterol	344 ± 63	393 ± 34	390 ± 54	433 ± 86	403 ± 83
HDL-C[d]	206 ± 56[b]	267 ± 26[b,c]	209 ± 34[c]	224 ± 22	220 ± 17
% HDL-C	60 ± 12	68 ± 8	54 ± 10	54 ± 11	56 ± 13
TG[e]	274 ± 136	222 ± 85	223 ± 62	274 ± 99	188 ± 69
			(mg/g liver)		
Hepatic lipids					
Total cholesterol	109 ± 10[b,c]	82 ± 8[c]	75 ± 12[b]	61 ± 13[c,x]	37 ± 4[x]
Free cholesterol	4 ± 0.6	3.5 ± 0.4	4 ± 0.4	4.2 ± 0.6	4.2 ± 0.5
Cholesterol esters	105 ± 10[b,c]	79 ± 7[b]	71 ± 12[b]	57 ± 13[c,x]	32 ± 4[x]

[a]Values are mean ± SD (n = 8).
[b,c]Means in a row sharing a common superscript are significantly different ($P < 0.05$) using analysis of variance and Scheffe's F-test.
[d]HDL-C, high-density lipoprotein-cholesterol.
[e]TG, triglycerides.
[x]Significantly different by Student's *t*-test ($P < 0.05$).

16:0 and high-16:0 intakes. Plasma HDL-C in the 12:0 + 14:0 group at six weeks was significantly higher than that in either the 18:1 group or the 14:0 + 16:0 group. The percent HDL-C (HDL-C/TC) in these groups tended to reflect the absolute amount of HDL-C. The plasma triglyceride concentration also increased significantly compared to the baseline value (157 ± 28), but no significant differences were observed between treatment groups, even though the high-fat, 16:0-rich diet tended to decrease triglyceride values.

Liver cholesterol. Livers from hamsters fed the high-fat, 16:0-rich diet (40% en as fat) weighed more than livers from the low-fat, 16:0-rich diet (Table 4), even though the TC and esterified cholesterol concentrations were significantly lower. By contrast, the 18:1-rich diet significantly increased hepatic cholesterol compared to other treatment groups. Free cholesterol was constant (about 4 mg/g) in all groups (Table 5).

Biliary lipids. When biliary lipid composition was expressed as molar ratios, the mol% bile acids was unaffected by the specific saturated fatty acid exchanges, whereas the mol% cholesterol was significantly lower in the 14:0 + 16:0 group. The biliary lipid composition was not altered by the low-fat vs. high-fat 16:0-rich diet comparison. The total biliary lipids concentration did not differ between treatment groups. The LI was highest in the 12:0 + 14:0 diet (3.8) and lowest in the 14:0 + 16:0 diet (1.6) (Table 6).

Bile acid profile. The total bile acid concentration (mM) was not affected by exchanging specific saturated fatty acids for 18:1 (Table 6). Neither the absolute concentration nor relative distribution of individual bile acids was remarkable except for glycocholate in the low-fat groups (Table 7), which was significantly higher in the 14:0 + 16:0 than the 12:0 + 14:0 and 18:1 diet groups. Taurochenodeoxycholate was the predominant bile acid in gallbladder bile and was unaffected by dietary treatments. Both taurocholate and glycocholate exhibited higher concentrations in the high-fat, 16:0-rich diet compared to the low-fat, 16:0-rich group. Total cholate was lowest in the 12:0 + 14:0 group, and total secondary bile acids were highest in this group, whereas total chenodeoxycholate

TABLE 6
Biliary Lipid Concentration, Lithogenic Index in Gallbladder Bile and Gallstone Incidence and Score in Hamsters Fed a Purified Diet Containing 0.4% Cholesterol and Different Dietary Fatty Acids for Six Weeks (Experiment 1)[a]

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
			(mM)		
Bile acids	110 ± 38	89 ± 18	133 ± 41	90 ± 20	128 ± 22
Phospholipids	26 ± 7	19 ± 6[b]	34 ± 8[b]	26 ± 9	42 ± 12
Cholesterol	28 ± 9	26 ± 11	19 ± 6	24 ± 12	30 ± 11
			(mol%)		
Bile acids	67 ± 6	66 ± 7	71 ± 5	65 ± 5	64 ± 6
Phospholipids	16 ± 4	14 ± 4	18 ± 3	19 ± 1	21 ± 4
Cholesterol	17 ± 3	20 ± 8[b]	11 ± 3[b]	17 ± 4	15 ± 2
			(g/dL)		
Total lipids	8.4 ± 2.5	6.8 ± 1.0	9.9 ± 2.6	7.4 ± 2.1	10.7 ± 2.2
Lithogenic index	2.9 ± 0.6	3.8 ± 1.7[b]	1.6 ± 0.4[b]	2.5 ± 0.4	2.0 ± 0.2
Gallstone incidence					
Cholesterol	1/8	5/8	3/8	8/8	3/8
Pigment	1/8	1/8	4/8	0/8	5/8
Gallstone score			(0–10)		
Cholesterol	0.2 ± 0.7	4.0 ± 3.6	1.4 ± 2.2	4.2 ± 2.5	2.2 ± 3.1
Pigment	0.4 ± 1.1	0.2 ± 0.7	0.7 ± 0.9	0	1.5 ± 1.9

[a]Values are mean ± SD (n = 8).
[b]Means in a row sharing a common superscript are significantly different ($P < 0.05$) using analysis of variance and Scheffe's F-test.

TABLE 7
Bile Acid Profile in Gallbladder Bile from Hamsters Fed a Purified Diet Containing 0.4% Cholesterol and Different Dietary Fatty Acids for Six Weeks (Experiment 1)[a]

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
			(mM)		
Taurocholate	28 ± 12	20 ± 6	26 ± 6	17 ± 4[x]	33 ± 6[x]
Glycocholate	21 ± 5[b]	12 ± 5[c]	36 ± 14[b,c]	21 ± 3[x]	37 ± 10[x]
Taurocheno	30 ± 12	30 ± 6	38 ± 14	31 ± 11	37 ± 6
Glycocheno	6 ± 2	5 ± 5	16 ± 18	7 ± 0.3	9 ± 4
Taurodeoxy	13 ± 14	13 ± 5	7 ± 3	9 ± 6	7 ± 2
Glycodeoxy	1 ± 1	2 ± 1	3 ± 5	1 ± 1	0.5 ± 0.5
Taurolitho	6 ± 8	6 ± 2	2 ± 2	4 ± 4	1 ± 1
Glycolitho	4 ± 2	4 ± 2	4 ± 1	4 ± 1	3 ± 3
			(% distribution)		
Taurocholate	26 ± 8	22 ± 6	21 ± 4	18 ± 1[x]	26 ± 2[x]
Glycocholate	21 ± 6	12 ± 5[b]	27 ± 7[b]	23 ± 7	29 ± 4
Taurocheno	27 ± 7	34 ± 6	29 ± 7	33 ± 9	29 ± 3
Glycocheno	6 ± 2	5 ± 6	10 ± 8	8 ± 2	7 ± 2
Taurodeoxy	10 ± 9	13 ± 4	5 ± 2	9 ± 4	5 ± 2
Glycodeoxy	1 ± 1	2 ± 1	2 ± 3	0.5 ± 1	0.4 ± 0.5
Taurolitho	5 ± 5	6 ± 2	1 ± 1	4 ± 3	1 ± 1
Glycolitho	4 ± 2	4 ± 2	4 ± 2	4 ± 3	3 ± 3
			(ratios)		
Cholate/cheno	1.5 ± 0.6	0.9 ± 0.3	1.3 ± 0.4	1.0 ± 0.3	1.5 ± 0.1
Glycine/taurine	0.5 ± 0.2	0.3 ± 0.1[b]	0.8 ± 0.4[b]	0.6 ± 0.3	0.6 ± 0.1
Primary/secd	5.1 ± 3.1	3.0 ± 0.9[b]	7.5 ± 2.7[b]	4.9 ± 1.7	11.3 ± 4.8
Hydrophobicity index	0.33 ± 0.05[b]	0.39 ± 0.03[b,c]	0.30 ± 0.03[c]	0.35 ± 0.02[x]	0.26 ± 0.03[x]

[a]Values are mean ± SD (n = 8).
[b,c]Means in a row sharing a common superscript are significantly different ($P < 0.05$) using analysis of variance and Scheffe's F-test.
[x]Significantly different by Student's *t*-test ($P < 0.05$).

TABLE 8
Bile Acid Pool Size and Fecal Bile Acid and Cholesterol Excretion in Hamsters Fed a Purified Diet Containing 0.4% Cholesterol and Different Dietary Fatty Acids for Six Weeks (Experiment 1)[a]

				16:0-rich	
	18:1-rich	12:0 + 14:0	14:0 + 16:0	Low-fat	High-fat
	(μmoles/kg body weight)				
Bile acid pool size	83 ± 10	114 ± 26	100 ± 23	108 ± 15	102 ± 12
Fecal excretion [g/three days (dry weight)]					
Feces	3.3 ± 0.7	2.8 ± 0.6	3.1 ± 0.4	3.0 ± 0.9	3.0 ± 0.9
Bile acids					
μmol/g feces[c]	1.5 ± 1.0[b]	2.2 ± 0.5	2.6 ± 1.0	3.3 ± 1.4[b]	3.7 ± 1.5
μmol/kg body weight	11 ± 7[b]	17 ± 6	20 ± 9	22 ± 7[b]	25 ± 8
Cholesterol					
μmol/g feces[c]	25 ± 7	25 ± 6	25 ± 4	33 ± 8[c]	44 ± 8[c]
μmol/kg body weight	210 ± 74	189 ± 62	187 ± 38	238 ± 71[c]	320 ± 103[c]

[a]Values are mean ± SD (n = 8). To convert μmoles to μg multiply bile acids by 491 and cholesterol by 387.
[b]Means in a row sharing a common superscript are significantly different ($P < 0.05$) using analysis of variance and Scheffe's F-test.
[c]Significantly different by Student's *t*-test ($P < 0.05$).

was lowest in the 18:1 group. The primary-to-secondary bile acid ratio was significantly higher during 14:0 + 16:0 than during 12:0 + 14:0 consumption and during high-fat, 16:0-rich intake than during low-fat, 16:0-rich consumption. However, the glycine-to-taurine ratio in the 12:0 + 14:0 group was significantly higher than the 14:0 + 16:0 group. The HI, a measure of the hydrophobic–hydrophilic balance among bile acids, was lowest in the high-fat, 16:0-rich diet group compared to the low-fat, 16:0-rich group. The HI was highest in the 12:0 + 14:0 diet group compared to the other low-fat groups (Table 7).

Gallstone incidence. Examination of gallbladders revealed striking differences in gallstone incidence between specific saturated fatty acid treatments (Table 6). Hamsters fed the low-fat, 16:0-rich diet proved to be the most susceptible, with eight of eight hamsters developing cholesterol gallstones. Hamsters fed the 18:1-rich diet proved least susceptible, with only one of eight hamsters developing gallstones. The 14:0 + 16:0 and the high-fat, 16:0-rich diets each produced cholesterol gallstones in three of eight hamsters, while the 12:0 + 14:0 diet generated stones in five of eight hamsters. However, both the low-fat, 16:0-rich and the 12:0 + 14:0 diets produced equally severe cholesterol gallstone scores. Mucin accumulation in gallbladder bile was often associated with cholesterol gallstones in diets containing 16:0 but was minimal in the 18:1-rich and 12:0 + 14:0 groups. When present, the thick, gel-like mucus was typically loaded with cholesterol crystals. No pigment stones were found in the low-fat, 16:0-rich diet group, while five of eight hamsters in the high-fat, 16:0-rich group developed pigment stones. The latter were observed in various stages of development, usually in bile without cholesterol crystals or stones. The early stage was characterized by cigar-shaped clumps or granules of inspissated mucin gel of yellow-green to amber coloration. The larger ones (0.2–0.5 mm) resembled jelly beans in shape and gooey consistency. Other more advanced forms represented glistening dark green-brown flakes of dried mucus, soft and somewhat pliable, often curled at the edges. The most mature stones were brownish-to-greenish black granules (up to 1.0 mm) with an irregular, pleomorphic configuration and a hard, friable, gritty texture.

Bile acid pool size. The bile acid pool size did not differ significantly among treatment groups although it tended to be smallest in the 18:1-rich group (Table 8).

Fecal bile acid and cholesterol excretion. Fecal bile acid excretion was lowest in the 18:1-rich group and significantly lower than the low-fat, 16:0-rich group. Fecal cholesterol excretion was significantly elevated in the high-fat, 16:0-rich diet group compared to the low-fat, 16:0-rich group, while no significant differences were observed between the low-fat treatments (Table 8).

Experiment 2

Plasma cholesterol and triglycerides. After six weeks of the purified diets containing graded amounts of 18:2, averages for body and organ weights were unaffected by dietary 18:2: total body, 128 g; liver, 6.5 g; cecum, 1.28 g; small intestine, 2.12 g. By six weeks, all four purified diets containing graded amounts of 18:2 elevated the plasma lipids above baseline, to an average TC of 424 mg/dL and TG of 444 mg/dL. No meaningful differences were observed.

Liver cholesterol and biliary lipids. The average hepatic cholesterol concentration [63 mg/g, 93% cholesteryl ester (CE)] was elevated equally by all diets, i.e., increasing the dietary 18:2 level had no effect. Adding graded amounts of 18:2 in the low-fat butter diet produced no significant differences

TABLE 9
Biliary Lipid Concentration, Lithogenic Index, Gallstone Incidence and Score in Hamsters Fed a Purified Diet Containing 5% Fat (butter-rich) and 0.4% Cholesterol and Graded Amounts of Linoleic Acid (safflower oil) for Six Weeks (Experiment 2)

	Dietary 18:2 percent energy (% en)[a]			
	0.3% en	0.6% en	0.9% en	1.2% en
		(mM)		
Bile acids	139 ± 19	106 ± 48	149 ± 30	142 ± 53
Phospholipids	25 ± 6	18 ± 6	21 ± 2	21 ± 9
Cholesterol	16 ± 8	14 ± 6	15 ± 7	9 ± 7
		(mol%)		
Bile acids	77 ± 4	75 ± 5	80 ± 5	83 ± 6
Phospholipids	14 ± 3	13 ± 2	11 ± 1	12 ± 3
Cholesterol	9 ± 4	11 ± 4	8 ± 4	5 ± 3
		(g/dL)		
Total lipids	9.4 ± 1.4	7.1 ± 2.9	9.5 ± 1.4	8.9 ± 3.3
Lithogenic index	1.7 ± 0.8	2.2 ± 0.6	1.3 ± 0.9	1.0 ± 0.4
Gallstone incidence				
Cholesterol	9/12	9/12	7/12	9/12
Pigment	4/12	3/12	4/12	1/12
Gallstone score		(0–10)		
Cholesterol	4.8 ± 4.1	3.8 ± 3.2	3.0 ± 3.3	3.1 ± 3.3
Pigment	0.6 ± 1.0	0.7 ± 1.5	1.2 ± 2.0	0.1 ± 0.3

[a]Values are mean ± SD (n = 12).

TABLE 10
Bile Acid Profile in Gallbladder Bile and Pool Size in Hamsters Fed a Purified Diet Containing 5% Fat (butter-rich) and 0.4% Cholesterol and Graded Amounts of Linoleic Acid (safflower oil) for Six Weeks (Experiment 2)

	Dietary 18:2 percent energy (% en)[a]			
	0.3% en	0.6% en	0.9% en	1.2% en
		(mM)		
Taurocholate	35 ± 13	24 ± 12	34 ± 10	37 ± 16
Glycocholate	42 ± 12	30 ± 24	46 ± 12	38 ± 17
Taurocheno	42 ± 8	33 ± 12	40 ± 7	42 ± 15
Glycocheno	37 ± 5	26 ± 13	36 ± 7	27 ± 11
Taurodeoxy	10 ± 2	10 ± 8	15 ± 5	10 ± 5
Glycodeoxy	4 ± 3	4 ± 2	8 ± 5	7 ± 3
Taurolitho	1 ± 2	1 ± 1	3 ± 2	3 ± 2
Glycolitho	2 ± 2	2 ± 3	5 ± 2	3 ± 1
		(% distribution)		
Taurocholate	20 ± 6	18 ± 4	18 ± 4	22 ± 4
Glycocholate	23 ± 5	23 ± 8	26 ± 4	23 ± 4
Taurocheno	24 ± 5	25 ± 8	21 ± 5	25 ± 4
Glycocheno	21 ± 3	20 ± 2	19 ± 3	16 ± 3
Taurodeoxy	6 ± 2	8 ± 3	8 ± 2	6 ± 1
Glycodeoxy	2 ± 2	3 ± 2	4 ± 3	4 ± 1
Taurolitho	1 ± 1	1 ± 1	2 ± 1	2 ± 1
Glycolitho	1 ± 1	2 ± 3	2 ± 1	2 ± 1
		(ratios)		
Cholate/cheno	1.0 ± 0.4	0.9 ± 0.3	1.0 ± 0.1	1.1 ± 0.3
Glycine/taurine	1.0 ± 0.1	0.9 ± 0.3	1.0 ± 0.3	0.8 ± 0.1
Hydrophobicity index	0.31 ± 0.05	0.33 ± 0.05	0.33 ± 0.02	0.31 ± 0.03
		(μmol/kg body weight)		
Bile acid pool size	88 ± 15	101 ± 14	127 ± 34	121 ± 27

[a]Values are mean ± SD (n = 12).

in biliary lipids or the LI, although the latter tended to decrease based on a tendency toward higher bile acids relative to bilary cholesterol associated with a slight rise in the cholate-to-cheno ratio (Tables 9 and 10). The glycine-to-taurine ratio was not affected, and the HI was similar in all treatment groups (Table 10).

Gallstone incidence. The amount of 18:2 in these low-fat diets did not modulate gallstone incidence, as 60–75%

of hamsters from each group developed cholesterol stones. However, the severity of cholesterol stone formation tended to decrease as the % en from 18:2 increased (Table 10).

DISCUSSION

Syrian hamsters are commonly used to study diet-induced cholelithiasis. Traditionally, induction of gallstones in hamsters utilized the EFA-deficient diet described by Dam (10) or diets containing a modest amount of butterfat with or without added cholesterol (3,20). Although a high intake of dietary cholesterol in a low-fat diet represents an anomaly from the practical, dietary point of view for humans, this manipulation definitely contributes to supersaturation of gallbladder bile and gallstone formation in the hamster model. In fact, a low level of dietary fat seems to be an important requirement for optimal stone formation (21). The secondary contribution of dietary fatty acids in this situation is less well-defined. Previous research has shown that certain dietary fats (7–10), and even specific fatty acids (22), alter lithogenesis. However, the reason for these associations is unclear.

The objective of our experiments was to determine whether varying the concentration of specific dietary fatty acids (as natural triglycerides) would alter bile lithogenicity and whether the absolute intake of the main fatty acid in question (16:0) would influence gallstone incidence. Accordingly, the different dietary treatments varied the intake of specific saturated and monounsaturated fatty acids, while safflower oil was added as necessary to assure an equal and adequate intake of 18:2 (EFA) at 1.2% en. The results reveal that (i) the monounsaturated fatty acid (18:1), as compared to the three saturated fatty acids (12:0,14:0,16:0), protects against gallstone formation in the cholesterol-fed hamster while increasing hepatic CEs and reducing bile acid excretion (synthesis); (ii) among the saturated fatty acids, 16:0 tended to be more lithogenic than 12:0 or 14:0, but specifically increasing 16:0 by higher fat intake reduced cholesterol gallstone formation; and (iii) the lithogenic effect of a low-fat diet rich in saturated fatty acids was not attributable to EFA deficiency since supplementation with EFAs (as safflower oil) did not alter lithogenicity.

Dietary fatty acids (as natural triglycerides) presumably influence all three major criteria involved in gallstone formation, i.e., cholesterol supersaturation of gallbladder bile, bile stasis due to reduced motility of the gallbladder, as well as nucleation and cholesterol crystal growth in the gallbladder (23). Biliary cholesterol supersaturation typically results from hypersecretion of cholesterol, hyposecretion of bile salts, or a combination of both. In the present study, the hepatic cholesterol concentration was elevated in all groups, but was highest in the group with the least stones (18:1-rich diet) and lowest in the two 16:0-groups (Experiment 1). Thus, hepatic cholesterol accumulation reflected the high dietary cholesterol content, but dietary monounsaturated fat exacerbated this effect, in keeping with previous data from cholesterol-fed hamsters (24). However, these fatty acids have minimal differential impact on hepatic cholesterol in the absence of dietary cholesterol (Hayes, K., and Dictenberg, J., unpublished data).

The increase in hepatic esterified cholesterol concentration with the 18:1-rich diet may reflect enhanced acyl cholesterol acyl transferase (ACAT) activity (24–26), which has been related to an increase in the hepatic low-density lipoprotein (LDL) receptor activity in cholesterol-fed hamsters (27). Saturated fatty acids reportedly suppressed LDL receptor activity and decreased the mass of hepatic CEs (27–29). One might anticipate that accumulation of hepatic cholesterol in the 18:1-rich diet group would increase biliary cholesterol secretion leading to supersaturated bile (LI greater than 1.0) and gallstones, or, conversely, that the lower hepatic cholesterol during 16:0 intake might reflect enhanced biliary cholesterol excretion and lead to gallstones. However, despite a relatively high LI in the 18:1-rich diet group, the incidence of gallstones was lowest in this group; and the high-fat, 16:0-rich diet (with no change in LI) actually decreased the incidence of stones while depressing the HI, suggesting that a low level of dietary fat is critical to gallstone formation and that higher concentrations of fat may actually be protective. These data confirm the lack of a specific relationship between elevated hepatic cholesterol concentration, biliary cholesterol saturation, and stone formation (15,30–32). Interestingly, although hamsters fed the low-fat, 16:0-rich diet were arguably the most severely afflicted with gallstones, those fed the low-fat, 14:0 + 16:0 diet, which is consistently lithogenic (7,15), had a slightly lower gallstone incidence and much lower severity of cholesterol stones than those fed the 12:0 + 14:0 diet. Coupled with the protective effect of added 16:0 in the high-fat, 16:0-rich diet, the inference is that 16:0 *per se* was not the initiating factor, but rather some aspect of its metabolism as the major fatty acid in a low-fat diet.

The cholate-to-cheno ratio tended to be lower in the two highly lithogenic diets (12:0 + 14:0 and low-fat, 16:0-rich diets) than in the other three diets, linking a low-cholate, high-chenodeoxycholate profile with stone formation. In general the primary bile acid profile was such that the lowest 18:1 intake (12:0 + 14:0-rich diet) resulted in the lowest total cholate and highest concentration of secondary bile acids, whereas high intake of 18:1 tended to assure a larger cholate pool and reduced cholesterol gallstones, even though bile acid synthesis and excretion were reduced. Thus, a low cholate/cheno ratio appears to be lithogenic for hamsters; and LDL receptor (LDL_r) activity, enhanced by 18:1 in the cholesterol-fed hamster (27), may be connected to the synthesis of cholate (1). A decrease in the cholate-to-cheno ratio has been observed previously in hamsters under a variety of gallstone-inducing dietary conditions (20,32–34), and the associated elevation in HI (33) has been hypothesized to depress 12 α-hydroxylase activity (32), which is essential for cholate synthesis (35).

Fecal bile acid and cholesterol excretion were highest in the 16:0-rich diet groups and were surprisingly greater ($P < 0.05$) than excretion of bile acids (and therefore synthesis) in the 18:1-rich group. However, despite these differences, the

similar LI and HI in these two groups imply that neither the bile lipid molar ratios nor bile acid profile per se was the definitive factor in the striking discrepancy in gallstone incidence between the two diets. The data also contrast with the increased susceptibility to cholesterol gallstones typically associated with a reduced bile acid pool. The high hepatic cholesteryl ester content associated with 18:1 intake would further suggest that the tendency of 18:1 to lower plasma cholesterol in these hypercholesterolemic hamsters, possibly by enhanced ACAT and LDL_r activities (24,26,27), does not promote sterol excretion but rather enhances cholesteryl ester deposition in tissues, which in itself may not be desirable.

Because a 5% butter diet is limited in EFA, it was unclear whether the lithogenic potential of butter was due to its saturated fatty acids or relative lack of either 18:2 or 18:1. Feeding graded amounts of 18:2 from the low level in 5% butter (0.3% en) to a level in excess of the EFA requirement (>1.0% en) under these lithogenic circumstances (Experiment 2) did not affect the incidence of cholesterol gallstones. Thus, marginal EFA depletion in the hamster fed butterfat and cholesterol does not appear to be an issue. However, adding 18:2 did seem to decrease pigment granules, at the highest 18:2 intake (1.2% en 18:2) and tended to cause progressive reduction in the LI.

In summary, the type and amount of dietary fatty acids impact cholesterol gallstone formation in hamsters, especially related to whether the fats (fatty acids) are consumed in high-fat or low-fat diets.

ACKNOWLEDGMENTS

The authors thank Andrzej Pronczuk for assisting with the formulation of the diets and for the fatty acid analysis of the diets.

REFERENCES

1. Hayes, K.C., Livingston, A., and Trautwein E.A. (1992) "Dietary Impact on Biliary Lipids and Gallstones," *Annu. Rev. Nutr. 12*, 299–326.
2. Liepa, G., Gorman, M.A., and Duffy, A.M. (1988) "The Use of Animals in Studying the Effects of Diet on Gallstone Formation," in *Comparative Animal Nutrition* (Beynen, A.C., and West, C.E., eds.) Vol. 6, pp. 149–173, S. Karger, Basel, Switzerland.
3. Tanimura, H. (1965) "Experimental Studies on the Etiology of Cholelithiasis," *Arch. Jpn. Chir. 34*, 1160–1179.
4. Wheeler, H.O. (1973) "Biliary Excretion of Bile Acids, Lecithin, and Cholesterol in Hamsters with Gallstones," *Gastroenterol. 65*, 92–103.
5. Bennion, L.J., and Grundy, S.M. (1978) "Risk Factors for the Development of Cholelithiasis in Man," *New Engl. J. Med. 299*, 1221–1227.
6. Maclure, K.M., Hayes, K.C., Colditz, G.A., Stampfer, M.J., and Willett, W.C. (1990) "Dietary Predictors of Symptom-associated Gallstones in Middle-aged Women," *Am. J. Clin. Nutr. 52*, 916–922.
7. Trautwein, E.A., Liang, J., and Hayes, K.C. (1993) "Cholesterol Gallstone Induction in Hamsters Reflects Strain Differences in Plasma Lipoproteins and Bile Acid Profiles," *Lipids 28*, 305–312.
8. Cohen, B.I., Mosbach, E.H., Ayyad, N., Miki, S., and McSherry, C.K. (1992) "Dietary Fat and Fatty Acids Modulate Cholesterol Cholelithiasis in the Hamster," *Lipids 27*, 526–532.
9. Shioda, R. (1965) "Experimental Studies or Gallstone Formation," *Arch. Jpn. Chir. 34*, 571–585.
10. Dam, H. (1971) "Determinants of Cholesterol Cholelithiasis in Man and Animals," *Am. J. Med. 51*, 596–613.
11. Weingard, K.W., and Daggy, B.P. (1990) "Quantification of HDL Cholesterol in Plasma from Hamsters by Differential Precipitation," *Clin. Chem. 36*, 575.
12. Hayes, K.C., Stephan, Z.F., Pronczuk, A., Lindsey, S., and Verdon, C. (1989) "Lactose Protects Against Estrogen-induced Pigment Gallstones in Hamsters Fed Nutritionally Adequate Purified Diets," *J. Nutr. 119*, 1726–1736.
13. Folch, J., Lees, M., and Stanley, G.H.S. (1957) "A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissue," *J. Biol. Chem. 226*, 497–509.
14. Rossi, S.S., Converse, J.L., and Hofmann, A.F. (1987) "High-Pressure Liquid Chromatographic Analysis of Conjugated Bile Acids in Human Bile: Simultaneous Resolution of Sulfated and Unsulfated Lithocholyl Amidates and The Commonly Conjugated Bile Acids," *J. Lipid Res. 28*, 589–595.
15. Trautwein, E.A., Liang, J., and Hayes, K.C. (1993) "Plasma Lipoproteins, Biliary Lipids and Bile Acid Profile Differ in Various Strains of Syrian Hamsters," *Comp. Biochem. Physiol. 104A*, 829–835.
16. Carey, M.C., and Small, D.M. (1978) "The Physical Chemistry of Cholesterol Solubility in Bile: Relationship to Gallstone Formation and Dissolution in Man," *J. Clin. Invest. 61*, 998–1026.
17. Heuman, D.M. (1989) "Quantitative Estimation of the Hydrophilic-Hydrophobic Balance of Mixed Bile Salt Solutions," *J. Lipid Res. 30*, 719–730.
18. Kim, J.C., and Chung, T.H. (1984) "Direct Determination of the Free Cholesterol and Individual Cholesteryl Esters in Serum by HPLC," *Korean J. Biochem. 16*, 69–77.
19. Suckling, K.E., Benson, G.M., Bond, B., Gee, A., Glen, A., Haynes, C., and Jackson, B. (1991) "Cholesterol Lowering and Bile Acid Excretion in the Hamster with Cholestyramine Treatment," *Atherosclerosis* 89, 183–190.
20. Cohen, B.I., Matoba, N., Mosbach, E.H., and McSherry, C. (1989) "Dietary Induction of Cholesterol Gallstones in Hamsters from Three Different Sources," *Lipids 24*, 151–156.
21. Hayes, K.C., Khosla, P., Kaiser, A., Yeghiazarians, V., and Pronczuk, A. (1992) "Dietary Fat and Cholesterol Modulate the Plasma Lipoprotein Distribution and Production of Pigment or Cholesterol Gallstones in Hamsters," *J. Nutr. 122*, 374–384.
22. Ayyad, N., Cohen, B.I., Mosbach, E.H., and Miki, S. (1992) "Palmitic Acid Enhances Cholesterol Gallstone Incidence in Sasco Hamsters Fed Cholesterol Enriched Diets," *Lipids 27*, 993–998.
23. Carey, M.C. (1989) "Formation of Cholesterol Gallstones: the New Paradigms," in *Trends in Bile Acid Research* (Paumgartner, G., Stiehl, A., and Gerok, W., eds.), pp. 259–281, Kluwer Academic Publishers, Dordricht/Boston/London.
24. Spady, D.K., Woollett, L.A., and Dietschy, J.M. (1993) "Regulation of Plasma LDL-Cholesterol Levels by Dietary Cholesterol and Fatty Acids," *Ann. Rev. Nutr. 13*, 355–381.
25. Ginsberg, R.L., Duane, W.C., and Flock, E.V. (1977) "Hepatic 3-Hydroxy-3-Methylglutaryl CoA Reductase Activity in Hamsters on a Lithogenic Diet," *J. Lab. Clin. Med. 89*, 928–936.

26. Ochoa, B., Gee, A., Jackson, B., and Suckling, K.E. (1990) "Regulation of Cholesteryl Ester Metabolism in the Hamster Liver," *Biochim. Biophys. Acta 1044*, 133–138.
27. Daumerie, C.M., Woollett, L.A., and Dietschy, J.M. (1992) "Fatty Acids Regulate Hepatic Low Density Lipoprotein Receptor Activity Through Redistribution of Intracellular Cholesterol Pools," *Proc. Natl. Acad. Sci. USA 89*, 10797–10801.
28. Woollett, L.A., Spady, D.K., and Dietschy, J.M. (1992) "Saturated and Unsaturated Fatty Acids Independently Regulate Low Density Lipoprotein Receptor Activity and Production Rate," *J. Lipid Res. 33*, 77–88.
29. Spady, D.K., and Dietschy, J.M. (1988) "Interaction of Dietary Cholesterol and Triglycerides in the Regulation of Hepatic LDL Transport in the Hamster," *J. Clin. Invest. 81*, 300–309.
30. Turley, S.D., Spady, D.K., and Dietschy, J.M. (1983) "Alteration of the Degree of Biliary Cholesterol Saturation in the Hamster and Rat by Manipulation of the Pools of Preformed and Newly Synthesized Cholesterol," *Gastroenterol. 84*, 253–264.
31. Spady, D.K., Turley, S.D., and Dietschy, J.M. (1983) "Dissociation of Hepatic Cholesterol Synthesis from Hepatic LDL Uptake Ad Biliary Cholesterol Saturation in Female and Male Hamsters of Different Ages," *Biochim. Biophys. Acta 753*, 381–392.
32. Gustafsson, B.F., Einarsson, K., and Gustafsson, J.A. (1975) "Influence of Cholesterol Feeding on Liver Microsomal Metabolism of Steroids and Bile Acids in Conventional and Germ-free Rats," *J. Biol. Chem. 250*, 8496–8502.
33. Trautwein, E.A., Siddiqui, A., and Hayes, K.C. (1993) "Modelling Plasma Lipoprotein-bile Lipid Relationships: Differential Impact of Psyllium and Cholestyramine in Hamsters Fed a Lithogenic Diet," *Metabolism 42*, 1531–1540.
34. Singhal, A.K., Finver-Sadowsky, J., McSherry, C.K., and Mosbach, E.H. (1983) "Effect of Cholesterol and Bile Acids on the Regulation of Cholesterol Metabolism in Hamster," *Biochim. Biophys. Acta 752*, 214–222.
35. Kuroki, S., Muramoto, S., Kuramoto, T., and Hoshita, T. (1983) "Effect of Feeding Cholesterol and Sitosterol on Hepatic Steroid 12 α-Hydroxylase Activity in Female Hamsters," *J. Pharm. Dyn. 6*, 551–557.

[Received February 16, 1994, and in revised form January 23, 1995; accepted February 27, 1995]

Evidence That Dietary Arachidonic Acid Increases Circulating Triglycerides[1]

Jay Whelan[a,*], Marc E. Surette[b], Bangyan Li-Stiles[a] and James W. Bailey[a]

[a]Department of Nutrition, University of Tennessee, Knoxville, Tennessee 37996-1900 and [b]Centre de Recherche Inflammation, Immunologie et Rheumatologie, Centre Hospitalier de l'Universite' Laval, Quebec, G1V4G2 Canada

ABSTRACT: Male Syrian hamsters and male CD-1 mice were fed diets supplemented with ethyl esters of oleic, linoleic, arachidonic, and eicosapentaenoic acids (1.1–1.5%, w/w) for 3–4 wk. Plasma and serum triglycerides were significantly higher in the arachidonic acid-supplemented animals compared to those in the other supplementation groups. Changes in serum insulin and glucose levels did not appear to be related to the changes in circulating triglycerides observed in the arachidonic acid-supplemented group. These data indicate that dietary arachidonic acid elevates circulating triglyceride levels compared to other unsaturated fatty acids in hamsters and mice by unknown mechanisms.

Lipids 30, 425–429 (1995).

Dietary polyunsaturated fatty acids (PUFA) are a group of heterogenous fatty acids which can possess dramatically diverse biological effects (1). Recent evidence demonstrates that dietary arachidonic acid (AA; 20:4n-6) possesses effects which are diametrically opposed to those observed with n-3 PUFA, and these effects appear to be related to the ability of dietary AA to alter tissue AA content and to abrogate the effects of n-3 PUFA (2–5).

One unequivocal effect of n-3 PUFAs, and in particular eicosapentaenoic acid (EPA; 20:5n-3), is their ability to lower circulating triglyceride levels (6). This is in comparison to n-6 PUFA derived from vegetable oils, i.e., linoleic acid (LA; 18:2n-6), which are relatively weak hypotriglyceridemic agents (6–9). However, the effects of dietary AA (desaturation and elongation product of LA) on circulating triglycerides remain unclear. Recently, it was reported that dietary AA may elevate plasma triglycerides, an effect which is opposite that of EPA (10). Based on the recent evidence for the opposing effects of dietary AA and EPA on circulating triglyceride levels and the limited research on the effects of dietary AA (3–5), these studies were designed to compare the effect that dietary AA has on circulating triglycerides with that of other dietary unsaturated fatty acids.

METHODS

Animals. Male Golden Syrian hamsters (~90 g) (Charles River, Wilmington, MA) or CD-1 male mice (~30 g) (HSD, Indianapolis, IN) were used in these studies. All animals were randomly divided and housed (hamsters: one per cage; mice: four per cage) on a 12-h dark/light cycle. All animals were fed standard laboratory pellet diets for one week, after which time they were fed experimental diets for three to four weeks as described previously (3,4). All animal procedures were approved by the University of Tennessee Animal Care and Use Committee (Knoxville, TN) and were in accord with the *NIH Guide for the Care and Use of Laboratory Animals (NRC 1985).*

Diets and experimental groups. The diets and experimental groups have been previously described in detail as part of larger studies (3,4) and, therefore, will only be briefly summarized. In Study 1, 24 male Syrian hamsters were randomly divided into four dietary groups (Table 1) (3). In the second study (Study 2), 49 male CD-1 mice were randomly divided into four dietary groups [n = 13: oleic acid (OA; 18:1n-9) group; n = 12: EPA, AA + EPA, and AA groups] (Table 1) (4). The animals in each study were fed identical diets [10% (w/w) fat] supplemented with various unsaturated fatty acid ethyl esters (1.1–1.5%, w/w), *viz.* OA, LA, AA, and EPA. Preparation and treatment of the diets are described elsewhere (3,4). Fatty acid analyses of the diets are presented in Tables 2 and 3 (3,4).

Triglyceride analysis. Animals were fasted overnight prior to blood collection. For hamster plasma triglycerides (Study 1), whole blood was drawn by cardiac puncture (nine parts blood and one part 38 g/L trisodium citrate) and centrifuged at 650 × *g* for 4 min. An aliquot of the plasma was analyzed for triglyceride content by the enzymatic measurement of triglyceride-derived glycerol (11) using the triglyceride analysis kit from Sigma (St. Louis, MO). The accuracy of the kit was validated using tripalmitin and glycerol standards. The coefficient of variation for multiple analyses performed on the same sample was 2%. For mouse serum triglycerides (Study 2), whole blood was drawn by cardiac

[1]This work was presented at the 78th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1994, Anaheim, CA.

*To whom correspondence should be addressed at Department of Nutrition, 229 Jessie Harris Building, University of Tennessee, Knoxville, TN.

Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; OA, oleic acid; PUFA, polyunsaturated fatty acids.

Copyright © 1995 by AOCS Press

TABLE 1
Unsaturated Fatty Acids Supplemented to the Diets of Study 1 (hamsters) and Study 2 (mice)[a,b]

	Dietary groups—Study 1[c]				Dietary groups—Study 2			
Fatty acid ethyl esters	OA group	LA group	EPA group	AA group	OA group	EPA group	AA + EPA group	AA group
18:1n-9	11.0	—	—	—	30	15	—	15
18:2n-6	—	11.0	—	—	—	—	—	—
20:4n-6	—	—	—	11.0	—	—	15	15
20:5n-3[d]	—	—	7.0	—	—	15	15	—

[a]The complete composition of the diets have been published elsewhere (Refs. 3,4). All diets contained all essential nutrients to maintain proper growth (based on AIN-76 diets) prior to supplementation with fatty acid ethyl esters.
[b]All components are g/kg diet as part of a 10% (w/w) fat diet.
[c]OA, oleic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid.
[d]The EPA diet (Study 1) contained 12 g n-3 polyunsaturated fatty acids ethyl esters/kg diet. The EPA ethyl ester used in Study 2 was 97% pure.

TABLE 2
Fatty Acid Composition of the Hamster Diets (Study 1)[a,b]

	Dietary groups			
Group	OA	AA	EPA	AA + EPA
16:0	41.4	41.0	41.0	40.0
18:1n-9	40.2	28.1	27.0	15.9
18:2n-6	18.3	18.1	18.0	17.9
20:4n-6	—[c]	12.8	—	12.3
20:5n-3	—	—	14.0	14.1

[a]See Reference 3. Abbreviations as in Table 1.
[b]All data are expressed as g/kg diet.
[c]Not detectable.

puncture and collected in tubes containing a gel clot activator (Bectin Dickison, Rutherford, NJ). Serum was isolated according to the manufacturer's instructions, and serum triglycerides were analyzed as stated previously.

Serum insulin and glucose. Serum insulin concentrations were determined by double antibody radioimmunoassay analysis (ICN Biomedical Inc., Costa Mesa, CA). Intra-assay coefficient of variation was 2.7%. Serum glucose concentrations were determined by the highly specific glucose oxidase method using an analysis kit from Sigma in which glucose oxidase and peroxidase, coupled with chromatogenic oxygen acceptor, are quantitated colorimetrically (12). The coefficient of variation for multiple analyses performed on the same sample was 3.4%.

Statistical analysis. The effects of dietary treatments were determined by analysis of variance. Comparisons between individual dietary groups were made using Fisher's protected least significance method using the Stat.View 512+ statistical package (Brain Power, Calabasas, CA).

TABLE 3
Fatty Acid Composition of the Mouse Diets (Study 2)[a,b]

	Dietary groups			
Fatty acid	OA	LA	AA	EPA
12:0	1.7	0.8	1.7	1.4
14:0	4.9	4.9	4.9	4.4
14:1	—[c]	—	—	0.3
16:0	19.3	19.5	18.9	18.3
16:1	1.2	1.2	1.2	1.2
18:0	6.0	6.8	6.4	6.1
18:1	47.2	36.2	36.7	37.2
18:2n-6	19.8	30.7	19.5	16.9
18:3n-3	—	—	—	0.5
18:4n-3	—	—	—	1.2
20:4n-6	—	—	10.7	—
20:5n-3	—	—	—	7.2
22:5n-3	—	—	—	0.6
22:6n-3	—	—	—	4.6

[a]See Reference 4. Abbreviations as in Table 1.
[b]All data are expressed as g/kg diet.
[c]Not detectable.

RESULTS

Weight gain and food intake were not significantly different among the different dietary groups in each respective study (Table 4).

Circulating triglyceride levels. Plasma triglyceride levels in hamsters fed the diet containing AA were 32 and 49% higher ($P < 0.05$) compared to animals in the LA and in the EPA dietary groups, respectively, and the difference approached significance ($P < 0.055$) compared to the OA group (Fig. 1). Similarly, serum triglyceride levels in mice fed the diet containing AA (devoid of EPA) were 24 and 30% higher ($P < 0.05$) compared to the OA and EPA group, respectively (Fig. 2). The serum triglyceride level in the AA + EPA group was not significantly different from the triglyceride level of any of the other dietary groups.

TABLE 4
Effects of Dietary Unsaturated Fatty Acids on Animal Weight Gain[a]

	Dietary groups			
	OA	EPA	AA + EPA	AA
Study 1	22.9 ± 3.6	21.7 ± 1.6	23.9 ± 1.7	22.1 ± 2.3
Study 2	21.5 ± 0.8	22.3 ± 2.0	22.6 ± 0.9	20.1 ± 1.6

[a]All data are expressed as mean ± SEM for six experimental values in Study 1 and 12–13 experimental values in Study 2. Weight gain of animals were reported as grams of weight gain/hamster/three weeks in Study 1 and grams of weight gain/four mice/four weeks in Study 2. Weight gains were not significantly different among dietary groups in each respective study. Abbreviations as in Table 1.

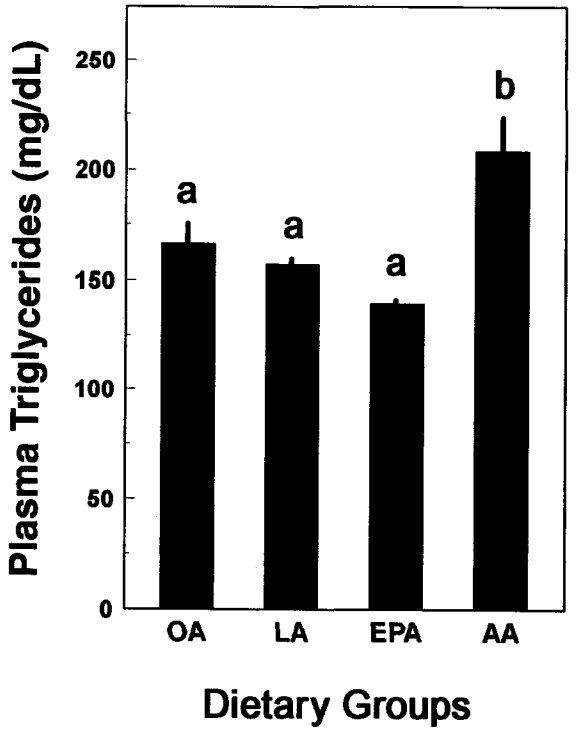


FIG. 1. The effect of dietary unsaturated fatty acids on plasma triglyceride levels in hamsters. Hamster diets were supplemented with one of the following unsaturated fatty acid ethyl esters: oleic acid (OA); linoleic acid (LA); eicosapentaenoic acid (EPA); arachidonic acid (AA). Plasma triglyceride levels were determined after three weeks on experimental diets. Data was pooled in each dietary group (n = 6) to generate means ± SEM. The AA group was significantly different from the LA and EPA groups at $P < 0.05$ and from the OA group at $P < 0.055$.

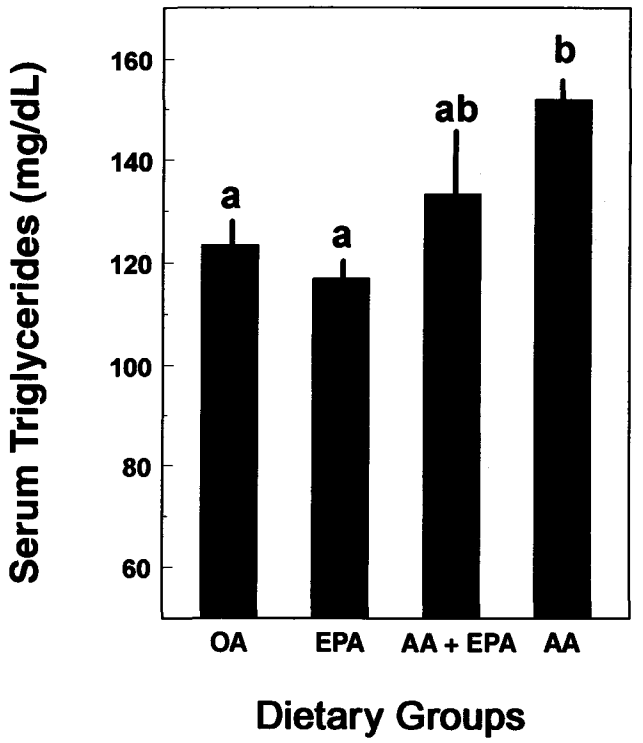


FIG. 2. The effect of dietary unsaturated fatty acids on serum triglyceride levels in mice. Diets were supplemented with the following unsaturated fatty acid ethyl esters: OA, EPA, AA. Data was pooled in each dietary group (n = 12 or 13) to generate means ± SEM. Values with different letters are significantly different at $P < 0.05$. Abbreviations as in Figure 1.

Serum insulin and glucose levels in mice (Study 2). Serum insulin levels were not significantly different among the different dietary groups (Table 5). Serum glucose levels in the AA group were not significantly different from the other dietary groups (Table 5), but those of the EPA and the AA + EPA dietary groups were significantly lower than those of the OA group.

DISCUSSION

A novel finding in this study was that the plasma triglyceride concentration was highest in the AA group compared to the other treatment groups. We observed statistically significant differences in the AA group compared to the LA and EPA groups ($P < 0.05$) and the OA group ($P < 0.055$) (Study 1). Our findings are supported by a study reporting that plasma triglycerides were 87% higher in streptozotocin-induced diabetic rats whose diets were supplemented with AA as compared with streptozotocin-treated controls (10). These more recent results are in contrast to an earlier study reporting that dietary AA did not appear to affect plasma triglyceride levels (13).

If dietary AA elevates plasma triglycerides and dietary n-3 PUFA are known to be hypotriglyceridemic, then the combination of AA and EPA (AA + EPA, Study 2) could nullify these divergent effects. We had previously established that an antithetic relationship existed between AA and EPA with regard to tissue fatty acid composition and eicosanoid production *in vitro* and *in vivo* (3,4). Therefore, a follow-up experiment was designed, in part, to further elucidate the relationship between dietary AA and circulating triglycerides and to explore the opposing effects that AA and EPA may have on circulating triglycerides.

The results observed in the first study were reproduced in the second study, where the AA-supplemented group (AA group, Study 2) had a significantly higher serum triglyceride concentration compared to the OA and EPA groups. The results from the two studies presented here suggest that dietary arachidonate possesses a triglyceride-elevating effect. When

TABLE 5
Effects of Dietary Unsaturated Fatty Acids on Serum Insulin and Glucose in Mice[a]

	Dietary groups			
	OA	EPA	AA + EPA	AA
Insulin (pmol/L)	57.1 ± 4.6[a]	53.2 ± 3.9[a]	50.6 ± 3.9[a]	52.5 ± 7.2[a]
Glucose (mg/dL)	154 ± 19[a]	123 ± 7[b]	124 ± 6[b]	145 ± 9[a,b]

[a]All data are expressed as mean ± SEM for 12–13 experimental values. Values in the same row with different letters are significantly different at $P < 0.05$. Abbreviations as in Table 1.

AA and EPA were included in the diet concomitantly (AA + EPA, Study 2), the triglyceride level in this group was midway between the triglyceride levels of the AA group and the EPA group, providing supporting (but not conclusive) evidence that AA and EPA may have opposing effects.

The effects of LA and EPA on plasma triglyceride levels, compared to those of OA (Fig. 1), were consistent with what has been reported in the literature (8,9,14). We observed that increasing chain length and unsaturation of dietary fatty acids resulted in a weak but progressive decline in plasma triglyceride concentrations, with the EPA group having the lowest value. However, although these changes were not statistically significant, they were also not surprising. Dietary LA possesses weak, but inconsistent, hypotriglyceridemic activity compared to saturated fatty acids and monosaturated fatty acids (7,15). n-3 PUFA, on the other hand, are very effective in lowering plasma triglyceride concentrations at dietary levels higher than those used in this study (6,16). When EPA was included in the diets (EPA group), the level of triglycerides were not different from the OA group, but the effect of EPA on triglyceride levels reflected similar quantitative and qualitative changes previously observed using the same animal model under similar experimental conditions (17). Therefore, our results are most likely due to the dietary level of n-3 PUFA used in the study (1.1%, w/w).

It has been reported that long-chain n-3 PUFA are poor substrates for β-oxidation, and that these PUFA, especially EPA, enhance mitochondrial and peroxisomal β-oxidation, suggesting n-3 PUFA may be affecting fatty acid availability for triglyceride synthesis (17–20). Dietary AA is also a poor substrate for β-oxidation (21), but recent evidence demonstrated that dietary AA has effects on β-oxidation which are in opposition to those observed with n-3 PUFA (22). It was reported that β-oxidation was significantly lower in AA-fed animals compared to animals consuming n-3 PUFA or OA-enriched diets. The authors suggested that the lower rates of fatty acid oxidation observed in the AA group favor triglyceride synthesis (22). However, dietary AA does not appear to significantly alter hepatic triglyceride secretion rates or plasma triglyceride fractional catabolic rates in rats (13), but some of the effects of AA on triglyceride secretion are difficult to interpret because of inconsistent results and the supraphysiological levels of AA used (1–20 mM) (23,24).

With respect to triglyceride synthesis, AA has been shown to be a potent modulator of lipogenesis by repressing the expression of a number of lipogenic genes (25–30); however, AA has also been shown to have contrasting effects on the activity of lipogenic enzymes such as glycerol-3-phosphate dehydrogenase (28,31,32). While all common PUFA have been shown to inhibit the expression of lipogenic genes, not all PUFA have hypotriglyceridemic properties. Therefore, further studies are required to more fully appreciate the relationship between dietary PUFA, circulating triglyceride levels, and expression of lipogenic genes.

Serum insulin levels were not different among the dietary groups (Study 2), indicating that dietary AA has little effect on circulating insulin levels. Serum insulin levels have been shown to be negatively correlated with AA content of muscle phospholipids in patients undergoing coronary artery surgery, but dietary AA intakes in these individuals were not reported (33). Similarly, dietary AA did not alter serum glucose levels (OA group vs. AA group). AA has been shown to reduce the responsiveness of the GLUT4 transporter to insulin in cultured 3T3-L1 adipocytes (34). However, in these same cells, AA had no effect on the maximal rate of glucose uptake as AA regulated GLUT4 and GLUT1 by altering their gene transcription and mRNA stability in opposite directions. In comparison, dietary fish oil supplementation, at a level of 20% of calories, improves insulin sensitivity in rats fed high-fat diets (59% of calories). However, serum insulin levels (Study 2) were not altered by EPA supplementation (3% of calories), and these results are consistent with those observed with non-diabetic healthy individuals fed diets containing fish oils (35,36). Although insulin levels remained unchanged, we nevertheless observed lower glucose levels in the EPA-containing groups. It has been reported that fish oil feeding improves insulin sensitivity in rats, possibly accounting for the observed results (37).

In summary, we observed that circulating triglyceride levels were elevated in hamsters and mice whose diets were supplemented with AA compared to animals whose diets were supplemented with OA, LA, or EPA, and these differences could not be explained by differences in circulating insulin or glucose levels. Although research evaluating the effects of dietary AA on lipid metabolism is limited, a review of the literature emphasizes the uncertainty of these effects, and this and other studies suggest that AA and n-3 PUFA could have effects which are in opposition (3–5,10,35). Our data and the data of Collier and Collier (10) provide the first evidence suggesting that dietary AA may elevate circulating triglyceride levels compared to other unsaturated fatty acids; however, the mechanisms responsible for these effects remain unclear.

ACKNOWLEDGMENTS

This work was supported in part by grants from the American Heart Association (Tennessee), Tennessee Agricultural Experiment Station, and a private research donation by Myron Pfeifer (Knoxville, TN).

REFERENCES

1. Kinsella, J.E., Broughton, K.S., and Whelan, J. (1990) *J. Nutr. Biochem. 1,* 123–140.
2. Whelan, J., Broughton, K.S., Surette, M.E., and Kinsella, J.E. (1992) *Lipids 27,* 85–88.
3. Whelan, J., Surette, M.E., Hardardottir, I., Lu, G.-P., Golemboski, K.A., Larsen, E., and Kinsella, J.E. (1993) *J. Nutr. 123,* 2174–2185.
4. Li, B.-Y., Birdwell, C., and Whelan, J. (1994) *J. Lipid Res.* 35, 1869–1877.
5. Mann, N.J., Warrick, G.E., O'Dea, K., Knapp, H.R., and Sinclair, A.J. (1994) *Lipids 29,* 157–162.
6. Harris, W.S. (1989) *J. Lipid Res. 30,* 785–807.

7. Harris, W.S., Connor, W.E. and McMurry, M.P. (1983) *Metabolism 32,* 179–184.
8. Nestel, P.J., Connor, W.E., Reardon, M.F., Connor, S., Wong, S., and Boston, R. (1984) *J. Clin. Invest. 74,* 82–89.
9. Berr, F., Geotz, A., Schreiber, E., and Paumgartner, G. (1993) *J. Lipid Res. 34,* 1275–1284.
10. Collier, F.M., and Collier, G.R. (1993) *Ann. N.Y. Acad. Sci. 683,* 365–366.
11. Printer, J.K., Hayashi, J.A., and Watson, J.A. (1967) *Archiv. Biochem. Biophys. 121,* 404–414.
12. Roabo, E., and Terkildsen, T.C. (1960) *Scand. J. Clin. Invest. 12,* 402–409.
13. Williams, M.A., Tinoco, J., Yang, Y.-T., Bird, M.I., and Hincenbergs, I. (1989) *Lipids 24,* 753–758.
14. Wardlaw, G.M., and Snook, J.T. (1990) *Am. J. Clin. Nutr. 51,* 815–821.
15. Goodnight, S.H., Harris, W.S., Connor, W.E., and Illingsworth, D.R. (1982) *Arteriosclerosis 2,* 87–113.
16. Bonna, K.H., Bjerre, K.S., and Nordoy, A. (1992) *Am. J. Clin. Nutr. 55,* 1126–1134.
17. Surette, M.E., Whelan, J., Broughton, K.S., and Kinsella, J.E. (1992) *Biochim. Biophys. Acta 1126,* 199–205.
18. Rustan, A.C., Christiansen, E.N., and Drevon, C.A. (1992) *Biochem. J. 283,* 333–339.
19. Steiner, S., Bremer, J., Berge, R.K., and Rustan, A.C. (1994) *J. Lipid Res. 35,* 1395–1404.
20. Dagnelic, P.C., Rietveld, T., Swart, G.R., Stijnen, T., and van den Berg, J.W.O. (1994) *Lipids 29,* 41–45.
21. Leyton, J., Drury, P.J., and Crawford, M.A. (1987) *Lipids 22,* 553–558.
22. Noit, I., Gresti, J., Biochot, J., Semporé, G., Durand, G., Bézard, J., and Clouet, P. (1994) *Lipids 29,* 481–489.
23. Ikeda, I., Murakami, J., Oka, T., Sugano, M., Yamada, H., Shimizu, S., Kawashima, H., Shinmen, Y., and Amashi, T. (1991) *Lipids 26,* 27–30.
24. Wong, S., Reardon, M., and Nestel, P. (1985) *Metabolism 34,* 900–905.
25. Landschulz, K.T., Jump, D.B., MacDougold, O.A., and Lane, M.D. (1994) *Biochem. Biophys. Res. Commun. 200,* 763–768.
26. Ntambi, J.M. (1992) *J. Biol. Chem. 267,* 10925–10930.
27. Tebbey, P.W., and Buttke, T.M. (1993) *Biochem. Biophys. Res. Commun. 194,* 862–868.
28. Salati, L.M., Adkins-Finke, B., and Clarke, S.D. (1988) *Lipids 23,* 36–41.
29. Clarke, S.D., and Jump D.B. (1993) *Annu. Rev. Nutr. 14,* 83–98.
30. Armstrong, M.K., Blake, W.L., and Clarke, S.D. (1991) *Biochem. Biophys. Res. Commun. 177,* 1056–1061.
31. Gaillard, D., Negrel, R., Lagarde, M., and Ailhaud, G. (1989) *Biochem. J. 257,* 389–397.
32. Chu, L.C., McIntosh, D.J., Hincebergs, I., and Williams, M.A. (1969) *Biochim. Biophys. Acta 187,* 573–575.
33. Borkman, M., Storlien, L.H., Pan, D.A., Jenkins, A.B., Chisholm, D.J., and Cambell, L.V. (1993) *N. Engl. J. Med. 328,* 238–244.
34. Tebby, P.W., McGowan, K.M., Stephens, J.M., Buttke, T.M., and Pekala, P.H. (1994) *J. Biol. Chem. 269,* 639–644.
35. Feskens, E.J.M., Bowles, C.H., and Kromhout, D. (1991) *Diabetes Care 14,* 935–941.
36. Stacpoole, P.W., Alig, J., Ammon, L., and Crockett, S.E. (1988) *Metabolism 38,* 946–956.
37. Storlien, L.H., Kraegen, E.W., Chisholm, D.J., Ford, G.L., Bruce, D.G., and Pascoe, W.S. (1987) *Science 237,* 885–888.

[Received October 18, 1994, and in revised form March 15, 1995; Revision accepted March 15, 1995]

Reduction of Hyperlipidemia in Hepatoma-Bearing Rats by Dietary Fish Oil

Masashi Kawasaki, Kazumi Yagasaki *, Yutaka Miura and Ryuhei Funabiki

Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183, Japan

ABSTRACT: The effect of dietary fish oil on serum lipid levels was studied by comparing it with dietary corn oil in Donryu rats subcutaneously implanted with an ascites hepatoma cell line (AH109A). The hepatoma-bearing rats exhibited hyperlipidemia charactarized by a rise in both serum cholesterol and triglyceride levels. Increased cholesterogenesis in the host liver and decreased steroid excretion into feces are suggested to be responsible for the hepatoma-induced hypercholesterolemia, and increased fatty acid mobilization from peripheral adipose tissues and decreased triglyceride clearance from the blood circulation are considered causes for the hepatoma-induced hypertriglyceridemia. Dietary fish oil reduced the hyperlipidemia in these animals, suppressed the hepatoma-induced increase in hepatic cholesterogenesis and fatty acid mobilization from adipose tissue. Dietary fish oil also tended to increase fatty acid oxidation in the liver. Such diverse effects of fish oil may lead to the reduction of the hepatoma-induced hyperlipidemia. These results suggest that studies on dietary fish oil may be warranted in patients with cancer-related hyperlipidemia.
Lipids 30, 431–436 (1995).

Various cancers affect serum lipoprotein profiles in humans (1,2). Hepatoma also induces abnormal serum lipid metabolism in humans (3,4) and in animals (5,6). Rats subcutaneously implanted with an ascites hepatoma line of AH109A cells show a notable decrease in high-density lipoprotein (HDL) fraction and an enormous increase in the very low density lipoprotein (VLDL) plus low-density lipoprotein (LDL) fraction (5) and a resultant hypertriglyceridemia (7) during growth of the hepatoma. Thus, AH109A-bearing rats provide us with an endogenously hyperlipidemic animal model that is quite distinct from the cholesterol (Ch)-loaded animals that have been widely used as an exogenously hypercholesterolemic model. Dietary fish oil (FO), rich in n-3 polyunsaturated fatty acids, has been well documented to suppress the levels of serum lipids, such as triglyceride (TG), phospholipid (PL), Ch, and nonesterified fatty acid (NEFA) in rats (8–13) and in hyperlipidemic patients (14). The present study examined the effect of dietary FO on serum lipids and various parameters related to lipid metabolism in both normal and hepatoma-bearing rats. Corn oil (CO), which is rich in n-6 linoleate, was employed as the control oil.

*To whom correspondence should be addressed at Department of Applied Biological Science, Tokyo Noko University, Saiwai-cho 3-5-8, Fuchu, Tokyo 183, Japan.

Abbreviations: ANOVA, analysis of variance; Ch, cholesterol; CO, corn oil; FA, fatty acid; FO, fish oil; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; HTGL, hepatic triglyceride lipase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NEFA, nonesterified fatty acid; PL, phospholipid; TG, triglyceride; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Animals and diets. Male Donryu rats (three weeks of age; NRC Haruna, Gunma, Japan) were individually housed in stainless-steel cages with wire bottoms and kept on a stock pellet diet (CE-2; CLEA Japan, Tokyo) in an air-conditioned room with a temperature of 22 ± 2°C, a relative humidity of 60 ± 5%, and an 8:00 a.m. to 8:00 p.m. light cycle. On the fourth day of the stock pellet feeding, rats with equal body weights were divided into two groups and for 23 d were fed a basal diet containing 20% casein (Oriental Yeast Co., Tokyo, Japan), 10% CO (Hayashi Chemicals Co., Tokyo, Japan), or FO (San-omega EPA-S18GA; the generous gift of NOF Corporation, Tokyo, Japan), 46.3% α-corn starch (Nihon Nosan Kogyo Co., Yokohama, Japan), 17% sucrose (Nissin Sugar Manufacturing Co., Tokyo, Japan), 3.5% mineral mixture (AIN composition; Nihon Nosan Kogyo Co.), 1% vitamin mixture (AIN composition; Nihon Nosan Kogyo Co.), 0.2% choline bitartrate (Wako Pure Chemical Industries, Osaka, Japan), and 2% cellulose powder (Oriental Yeast Co.). The FO contained 0.3% vitamin E, so the CO diet was also supplemented with extra vitamin E (Riken E Oil 800; Riken Vitamin Co., Tokyo, Japan) in order to make the vitamin E content of both diets equal (350 mg/kg diet). No side effects have been noted at this dose of vitamin E in rats (15). The fatty acid (FA) composition of each oil is shown in Table 1. There was no Ch in CO, and 0.5% in FO, whereas the β-sitosterol content was 0.3% in CO with none in FO. Thus, the CO diet contained 0.03% β-sitosterol, whereas the FO diet contained 0.05% Ch. An enzymatic assay employed for the determination of fecal neutral sterols reacts with both Ch and β-sitosterol (38.4% of Ch). After feeding the experimental diets for 23 d, rats of either the CO group or the FO group were again divided into two groups with equal body weights. Half the rats of each oil group received a subcutaneous implantation of 5×10^5 AH109A cells (provided by SRL, Tokyo, Japan) in the back to produce a solid hepatoma (5), and the other half

Copyright © 1995 by AOCS Press

TABLE 1
Fatty Acid Composition of Corn and Fish Oils

Fatty acid	Total fatty acids (%)	
	Corn oil	Fish oil
14:0	trace	6.1
16:0	11.5	14.2
16:1	trace	7.1
18:0	2.3	2.0
18:1	31.3	11.9
18:2n-6	53.3	1.4
18:3n-3	trace	1.0
18:4n-3	trace	3.6
20:5n-3	trace	17.7
22:6n-3	trace	11.8

received only a sham injection of phosphate-buffered saline (0.5 mL/rat) and were designated as normal rats (day 0). Rats of each group were kept for a further 14 d on the same test diets. Water and each diet were available at all times. Animals were deprived of their diet at 9:00 a.m. on day 14, but allowed free access to water until killing (which was performed 4 h later by decapitation). Blood was collected and left to clot at room temperature to obtain serum. The liver, epididymal adipose tissue, cardiac muscle, gastrocnemius, and solid hepatoma were quickly removed, washed with cold 0.9% NaCl, and blotted on filter paper. Aliquots of these tissues were frozen in liquid nitrogen and stored at −70°C until analyzed.

Lipid analyses. Total lipids were extracted according to the procedure of Folch *et al.* (16) from the liver and solid hepatoma. After portions of the chloroform phase had been dried under nitrogen, Ch (17), TG (18), and PL (19) were determined as previously described (20). The serum TG and PL levels were also determined as described previously. The serum NEFA level was measured by the method of Kushiro *et al.* (21). The serum lipoproteins were separated into HDL and VLDL + LDL fractions by the precipitation method (5). The total Ch of unfractionated serum (T-Ch) and HDL (HDL–Ch) were determined by an enzymatic method using a cholesterol C-test kit (Wako Pure Chemical Industries), and the difference between T-Ch and HDL-Ch was regarded as (VLDL + LDL)-Ch.

Measurement of tissue lipolytic activities. Lipoprotein lipase (LPL) was extracted from epididymal adipose tissue, cardiac muscle, and gastrocnemius by the method of Noguchi *et al.* (22); the tissues weighing 300 mg were homogenized in 0.2 M Tris-HCl buffer (pH 8.5) containing 10 U/mL of sodium heparin (Wako Pure Chemical Industries). Hepatic TG lipase (HTGL) was also extracted from the liver and the solid hepatoma in the same way. Lipase (LPL and HTGL) activities were then estimated as described previously (23,24). Briefly, 0.1 mL of the abovementioned enzyme source was mixed with 0.1 mL of substrate solution (arabic gum-emulsified Tris-HCl buffer, 0.2 M and pH 8.0, containing rat serum to supply adequate apolipoprotein C-II as an LPL activator for LPL, and 0.1 M and pH 8.5 for HTGL) containing 925 Bq (55,500 dpm)/μmol/assay of [carboxyl-^{14}C]triolein (original specific radioactivity, 4.1 GBq/mmol; NEN Research Products, Boston, MA). The mixture was incubated at 37°C for one hour, followed by the extraction and counting of hydrolyzed [^{14}C]oleic acid. Hormone-sensitive lipase (HSL) activity in adipose tissue was also determined. From epididymal adipose tissue, HSL was extracted with two volumes of 0.2 M phosphate buffer (pH 7.0) by homogenization (25). The enzyme suspension (0.1 mL) previously incubated with 1 M NaCl for 10 min was mixed with 0.1 mL of substrate solution (arabic gum-emulsified 0.2 M phosphate buffer, pH 7.0) containing 925 Bq/μmol/assay of [carboxyl-^{14}C]triolein and the mixture was incubated at 30°C for one hour. At the end of the incubation period, hydrolyzed [^{14}C]oleic acid was extracted, and radioactivity was counted after adding Insta-Gel (Packard Instrument Co., Inc., Meriden, CT). One unit of lipase activity was defined as 1 μmol of FA released per hour.

Hepatic FA oxidation. FA oxidation was estimated using liver slices (26). Liver slices weighing 200 mg were placed in 2 mL of Krebs-Ringer phosphate buffer (pH 7.4) containing 37 kBq/μmol/assay of [1-^{14}C]palmitic acid (original specific radioactivity 310 MBq/mmol; NEN Research Products). Each tube was gassed with 100% O_2, connected with a rubber tube (18 mm i.d.) to a counting vial which held circular filter paper (No. 131, 24 mm in diameter; Toyo Roshi Co., Tokyo, Japan), saturated with 100 μL of Protosol (NEN Research Products), and incubated at 37°C for two hours. The incubation was terminated with 1 mL of 8% $HClO_4$ *via* the rubber tube with a needle, and thereafter each tube was incubated for another hour to absorb $^{14}CO_2$ to Protosol. The residual layer was extracted twice with petroleum ether to remove traces of [^{14}C]palmitic acid, and an aliquot of the aqueous phase was counted (acid-soluble oxidation products: ketone bodies). Radioactivity was measured after adding Insta-Gel.

Hepatic lipid syntheses. Total FAs and Ch syntheses were measured using liver slices (26,27). Liver slices weighing 100–120 mg were placed in 1 mL of Krebs-Ringer phosphate buffer (pH 7.4) containing 37 kBq/μmol/assay of [1-^{14}C]-acetic acid (sodium salt, original specific radioactivity 2.04 GBq/mmol; Amersham International plc, Buckinghamshire, United Kingdom). Each tube was gassed with 100% O_2, stoppered tightly with a screw cap, and incubated at 37°C for two hours. At the end of the incubation period, 1 mL of 15% ethanolic KOH was added, and the mixture was saponified at 75°C for two hours. Nonsaponifiable lipids were extracted three times with petroleum ether, and digitonin-precipitable sterols were formed, isolated, washed, dissolved in methanol, and counted after addition of a toluene-based scintillator. The residual aqueous layer was acidified to pH 2 with concentrated HCl, and total FAs were extracted three times with petroleum ether. Pooled extracts were dried under nitrogen, dissolved in chloroform, and washed twice with water. The chloroform layer was quantitatively transferred to a scintillation vial, dried, and counted as mentioned previously.

Fecal steroid excretion. Feces were collected for two days before sacrifice (days 12–14). Neutral sterols and bile acids

TABLE 2
Initial Body Weight, Food Intake, Body Weight Gain, Weights and Lipid Contents of Liver and Hepatoma in Normal and in Hepatoma-Bearing Rats[a]

Measurement	Normal		Hepatoma-bearing		ANOVA[b]
	Corn oil	Fish oil	Corn oil	Fish oil	
Initial body weight (g)	243.5 ± 11.9	240.3 ± 7.2	244.0 ± 6.7	240.9 ± 8.2	
Food intake (g/14 d)	276.8 ± 15.0[c]	268.8 ± 10.0[c]	183.8 ± 11.3[d]	186.3 ± 10.2[d]	S
Body weight gain (g/14 d)	80.0 ± 5.4[c]	74.8 ± 3.2[c]	29.9 ± 9.3[d]	39.9 ± 9.2[d]	S
Liver weight (g)	12.2 ± 0.8[c]	12.4 ± 0.6[c]	10.1 ± 0.7[d]	11.3 ± 0.6[c,d]	S
Hepatoma weight (g)	—	—	40.5 ± 5.1	36.2 ± 3.2	
Liver lipid content (μmol/g liver)					
Triglyceride	34.9 ± 6.4[c]	12.5 ± 3.2[d]	9.2 ± 2.4[d]	4.7 ± 0.7[d]	S, O, S × O
Phospholipid	37.1 ± 1.2	38.2 ± 1.8	35.8 ± 3.0	34.0 ± 2.3	
Cholesterol	4.1 ± 0.5	3.6 ± 0.0	4.1 ± 0.3	3.4 ± 0.3	
Hepatoma lipid content (μmol/g hepatoma)					
Triglyceride	—	—	2.0 ± 0.2	1.6 ± 0.1	
Phospholipid	—	—	8.1 ± 0.4[f]	10.5 ± 0.5[e]	O
Cholesterol	—	—	5.2 ± 0.3	5.7 ± 0.3	

[a]Each value represents the mean ± standard error of six rats.
[b]When the F value for either hepatoma state (S) effect, dietary oil (O) effect, or both interaction (S × O) was significant at $P < 0.05$, the means were inspected. ANOVA, analysis of variance.
[c,d]Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.
[e,f]Values not sharing a common letter are significantly different at $P < 0.05$ by Student's *t*-test.

were extracted from dried and ground feces after saponification according to the procedure of Yamanaka *et al.* (28). Fecal 3β-hydroxy neutral sterols and 3α-hydroxy bile acids were determined by enzymatic methods (29), using commercial kits as described previously (30).

Statistical analysis. Results were expressed as mean ± standard error. Statistical analysis was carried out by two-way analysis of variance (ANOVA) followed by Duncan's multiple-range test (31). In some cases (Table 2), Student's *t*-test was employed after one-way ANOVA. The correlation coefficient was determined by least square analysis.

RESULTS

Both food intake and body weight gain during the 23 d of preliminary feeding were almost the same between the CO and FO diet groups (data not shown). Table 2 shows food intake, body weight gain, liver and solid hepatoma weights, and lipid contents in the liver and hepatoma 14 d after AH109A implantation [hepatoma-bearing (H) groups] or phosphate-buffered saline injection [normal (N) groups]. Food intake, body weight gain, and liver weight were reduced by bearing hepatoma (N-CO vs. H-CO), but FO as compared with CO had no significant effects on these parameters in either the normal (N-CO vs. N-FO) or the hepatoma-bearing (H-CO vs. H-FO) state. Feeding the FO diet did not influence hepatoma weight, although a slight (11%) decrease was seen (H-CO vs. H-FO). The liver TG content was decreased by both bearing hepatoma (N-CO vs. H-CO) and feeding FO (N-CO vs. N-FO), whereas the liver PL and Ch contents were not affected by either diet or hepatoma. The hepatoma PL content was increased, but the TG and Ch contents were not affected by feeding FO (H-CO vs. H-FO).

Changes in the serum lipid levels are illustrated in Figure 1. The serum TG, PL, NEFA, T-Ch, and (VLDL + LDL)-Ch levels were significantly higher in the hepatoma-bearing rats than in the normal rats, irrespective of dietary oils (N-CO vs.

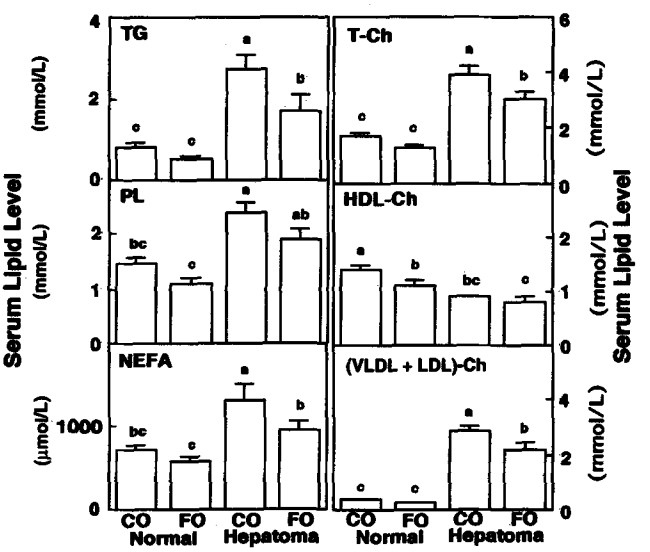


FIG. 1. Effects of types of dietary oils on serum lipid levels in normal and in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical bars indicate standard errors. When the F value for either hepatoma state (S) effect, dietary oil (O) effect, or both interaction (S × O) was significant at $P < 0.05$, the means were inspected by Duncan's multiple-range test. Values not sharing a common letter are significantly different at $P < 0.05$. The F values for S and O of triglyceride (TG), phospholipid (PL), nonesterified fatty acid (NEFA), total cholesterol (T-Ch), total cholesterol of high-density lipoprotein (HDL-Ch) and (VLDL + LDL)-Ch (the difference between T-Ch and HDL-Ch) level were all significant at $P < 0.05$. CO, corn oil; FO, fish oil.

H-CO, N-FO vs. H-FO). The reductive effect of FO on these lipid levels was weak in the normal state (N-CO vs. N-FO). However, FO, as compared with CO, significantly suppressed the serum TG, NEFA, T-Ch, and (VLDL + LDL)-Ch levels but did not significantly suppress the PL level in the hepatoma-bearing state (H-CO vs. H-FO). Conversely, the serum HDL-Ch level was significantly lower in the hepatoma-bearing state than in the normal state, irrespective of dietary oils (N-CO vs. H-CO, N-FO vs. H-FO). In the normal state, FO more than CO significantly decreased HDL-Ch level (N-CO vs. N-FO), although FO did not further decrease the HDL-Ch level in the hepatoma-bearing state (H-CO vs. H-FO).

Table 3 shows activities of LPL, HTGL, and HSL in adipose tissue and in other tissues. The LPL activity in adipose tissue, cardiac muscle, and gastrocnemius, and the HTGL activity in the liver were notably lower in the hepatoma-bearing state than in the normal state (N-CO vs. H-CO, N-FO vs. H-FO). Dietary FO, as compared with CO, had no significant influence on these lipase activities in either the normal or the hepatoma-bearing state, except for the LPL activity of adipose tissue in the normal state that was significantly enhanced by feeding dietary FO (N-CO vs. N-FO). When LPL activity was expressed as U per whole tissue, the enzyme activity in adipose tissue was also lower in the hepatoma-bearing state than in the normal state. However, the stimulatory action of FO on LPL activity weakened, especially in the normal state. The HTGL activity in hepatoma was unaffected by dietary FO (H-CO vs. H-FO). In contrast to the LPL activity, the adipose tissue HSL activity was significantly enhanced by bearing hepatoma (N-CO vs. H-CO). Dietary FO showed no significant effect on the HSL activity in comparison with dietary CO in the normal and in the hepatoma-bearing states (N-CO vs. N-FO, H-CO vs. H-FO).

FA oxidation and lipid (FA and Ch) syntheses by host liver slices are shown in Table 4. The production of $^{14}CO_2$ was unchanged among the four groups. However, the production of [^{14}C]ketone bodies and, hence, total FA oxidation in the hepatoma-bearing state, were significantly higher than in the normal state (N-CO vs. H-CO, N-FO vs. H-CO). Dietary FO showed no significant enhancement in the production of [^{14}C]ketone bodies and total FA oxidation in both the normal and the hepatoma-bearing states, although slight increases (18–20%) were observed (N-CO vs. N-FO, H-CO vs. H-FO). Neither hepatoma nor dietary FO affected FA synthesis by liver slices. On the other hand, the hepatic Ch synthesis was significantly stimulated by hepatoma implantation (N-CO vs. H-CO), and this rise in the hepatoma-bearing state was suppressed by dietary FO when compared with dietary CO (H-CO vs. H-FO).

Fecal steroid excretion is also shown in Table 4. Dry weight of feces was significantly lower in the hepatoma-bearing state than in the normal state (N-CO vs. H-CO, N-FO vs. H-FO). Excretion of both neutral sterols and bile acids was reduced by bearing tumor (N-CO vs. H-CO). Dietary FO as compared with CO showed no significant effect on their excretion, except for neutral sterols in the normal state where dietary FO significantly suppressed their excretion into feces (N-CO vs. N-FO).

DISCUSSION

Donryu rats receiving a subcutaneous implantation of ascites hepatoma line of AH109A cells exhibited hyperlipidemia, and dietary FO as compared with CO was demonstrated to reduce the hyperlipidemia in these animals. The HDL-Ch level was decreased slightly but significantly by hepatoma implantation, but dietary FO did not further accelerate the hepatoma-induced reduction of the HDL-Ch level despite the fact that FO decreased the serum level of this antiatherogenic lipoprotein in the normal state. The hypocholesterolemic and hy-

TABLE 3
Activities of Tissue Lipoprotein Lipase, Hepatic Triglyceride Lipase, and Hormone-Sensitive Lipase in Normal and in Hepatoma-Bearing Rats[a]

Measurement	Normal		Hepatoma-bearing		ANOVA[b]
	Corn oil	Fish oil	Corn oil	Fish oil	
Lipoprotein lipase activity					
Epididymal adipose tissue					
(U/g tissue)	7.5 ± 0.7[d]	10.3 ± 1.0[c]	2.8 ± 0.5[e]	3.4 ± 0.3[e]	S, O
(U/whole tissue)	18.8 ± 2.4[c]	20.6 ± 2.7[c]	3.6 ± 0.5[d]	4.1 ± 1.2[d]	S
Cardiac muscle (U/g tissue)	13.5 ± 0.6[c]	13.6 ± 0.4[c]	6.7 ± 1.2[d]	7.0 ± 1.1[d]	S
Gastrocnemius (U/g tissue)	2.1 ± 0.2[c]	2.8 ± 0.2[c]	1.3 ± 0.4[d]	1.2 ± 0.3[d]	S
Hepatic triglyceride lipase activity					
Liver (U/g tissue)	2.3 ± 0.2[c]	2.4 ± 0.1[c]	1.2 ± 0.1[d]	1.2 ± 0.2[d]	S
Hepatoma (U/g tissue)	—	—	1.3 ± 0.2	1.4 ± 0.2	
Hormone-sensitive lipase activity					
Epididymal adipose tissue					
(U/g tissue)	0.9 ± 0.1[d]	0.9 ± 0.1[d]	1.2 ± 0.2[c]	1.0 ± 0.1[c,d]	S

[a]Each value represents the mean ± standard error of six rats.
[b]When the F value for either hepatoma state (S) effect, dietary oil (O) effect, or both interaction (S × O) was significant at $P < 0.05$, the means were inspected. ANOVA, analysis of variance.
[c–e]Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

TABLE 4
Hepatic Fatty Acid Oxidation, Lipid Syntheses, and Fecal Steroid Excretion in Normal and in Hepatoma-Bearing Rats[a]

Measurement	Normal		Hepatoma-bearing		ANOVA[b]
	Corn oil	Fish oil	Corn oil	Fish oil	
Hepatic fatty acid oxidation (dpm 10^{-4}/2 h/g liver)					
Total	50.7 ± 4.2[e]	60.9 ± 6.0[d,e]	72.3 ± 3.3[c,d]	85.1 ± 8.5[c]	S
CO_2	4.0 ± 0.4	4.7 ± 1.0	4.2 ± 0.4	4.8 ± 0.6	
Ketone bodies	46.7 ± 3.8[e]	56.3 ± 5.4[d,e]	68.2 ± 3.3[c,d]	80.3 ± 8.3[c]	S
Hepatic lipid syntheses (dpm 10^{-4}/2 h/g liver)					
Fatty acids	4.9 ± 0.6	4.3 ± 0.8	4.9 ± 1.3	4.0 ± 1.1	
Cholesterol	2.5 ± 0.6[d]	2.0 ± 0.3[d]	7.6 ± 1.3[c]	4.8 ± 1.2[d]	S
Fecal weight (g/2 d)	2.0 ± 0.2[c]	2.1 ± 0.1[c]	0.7 ± 0.1[d]	1.1 ± 0.3[d]	S
Steroid excretion (μmol/2 d)					
Neutral sterols	45.9 ± 5.9[c]	22.0 ± 1.2[d]	15.4 ± 2.8[d]	13.5 ± 2.0[d]	S, O, S × O
Bile acids	18.2 ± 1.9[c]	19.4 ± 2.6[c]	8.2 ± 1.1[d]	9.5 ± 1.2[d]	S

[a]Each value represents the mean ± standard error of six rats.
[b]When the F value for either hepatoma state (S) effect, dietary oil (O) effect, or both interaction (S × O) was significant at $P < 0.05$, the means were inspected. ANOVA, analysis of variance.
[c–e]Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

potriglyceridemic actions of FO were prominent in the tumor-bearing state than in the tumor-free state (Fig. 1). The ingestion of FO may, therefore, possibly have a similar effect in patients with cancer-related hyperlipidemia.

The present results show that at least two factors, hepatic cholesterogenesis and fecal steroid excretion, are responsible for the induction of hypercholesterolemia by AH109A implantation. The Ch synthesis by liver slices from the hepatoma-bearing rats was threefold greater than those from the normal rats (Table 4). This finding is in good agreement with our previous results showing cholesterogenesis *in vivo* from ^3H_2O in the liver of hepatoma-bearing rats was twice that in the liver of normal rats (32). Another factor, fecal steroid excretion, was reduced by hepatoma bearing. Dietary FO suppressed the hepatoma-induced enhancement of host liver cholesterogenesis, while the oil demonstrated no influence on the hepatoma-induced reduction of steroid excretion. The CO and FO used here are actually in the TG form, and there was little neutral 3β-hydroxysterols in both oils and, hence, diets, i.e., less than 0.05% in both diets. In rats fed Ch-enriched (0.5%) diets, β-sitosterol was reported to increase fecal excretion of neutral sterols at the dietary level of 0.5% or more (33). In the present study, such small amounts (less than 0.05%) of dietary neutral sterols, Ch and β-sitosterol, are therefore unlikely to be responsible for the results. The hypocholesterolemic effect of dietary FO in the hepatoma-bearing state may thus mainly be due to its inhibitory action on hepatic cholesterogenesis.

Increased FA mobilization from peripheral adipose tissue and decreased TG clearance from the blood circulation are likely to contribute to the hepatoma-induced hypertriglyceridemia. The serum NEFA level was elevated in hepatoma-bearing rats (Fig. 1), as has been reported in rats (13) and in cancer patients (34). The high level of serum NEFA in the hepatoma-bearing state coincides with the rise in adipose tissue HSL activity (Table 3). There was a positive correlation between the serum NEFA and TG levels ($r = 0.84$, $P < 0.01$, $n = 12$ in N-CO and H-CO groups). The rise in the serum NEFA level would augment a supply of fatty acyl-CoA to be esterified to PL as well as TG, and hence formation and secretion of VLDL from the liver, although a portion of the increased fatty acyl-CoA might be rapidly oxidized in the livers of the hepatoma-bearing rats (Table 4). Tissue LPL and HTGL activities decreased in the hepatoma-bearing state, possibly leading to a reduced clearance of serum TG and, hence, to hypertriglyceridemia. The reduction of LPL activity is also observed in animals bearing some other tumors (22,35). Dietary FO suppressed FA mobilization and tended to enhance FA oxidation in the livers of hepatoma-bearing rats. The hypotriglyceridemic action of dietary FO is suggested to be a result of integration of these diverse effects in the animals.

Intriguing questions arise from these results: Why do the HDL level, LPL activity, and fecal steroid excretion decrease in the hepatoma-bearing state, and why does cholesterogenesis increase in the host liver of hepatoma-bearing rats? Further studies are needed to answer these questions and to clarify the exact mechanisms involved in the action of FO.

ACKNOWLEDGMENT

This work was supported by a grant from the Foundation of Skylark Food Science Institute, Japan.

REFFERENCES

1. Barclay, M., Skipski, V.P., Terebus-Kekish, O., Greene, E.M., Kaufman, R.J., and Stock, C.C. (1970) *Cancer Res. 30,* 2420–2430.

2. Nydegger, U.E., and Butler, R.E. (1972) *Cancer Res. 32*, 1756–1760.
3. Alsabti, E.A.K. (1979) *Oncology 36*, 11–14.
4. Hirayama, C., Yamanishi, Y., and Irisa, T. (1979) *Clin. Chim. Acta 91*, 53–57.
5. Irikura, T., Takagi, K., Okada, K., and Yagasaki, K. (1985) *Lipids 20*, 420–424.
6. Clark, R.W., and Crain, R.C. (1986) *Cancer Res. 46*, 1894–1903.
7. Yagasaki, K., Machida, M., and Funabiki, R. (1986) *J. Nutr. Sci. Vitaminol. 32*, 643–651.
8. Ribeiro, A., Mangeney, M., Cardot, P., Loriette, C., Rayssiguier, Y., Chambaz, J., and Bereziat, G. (1991) *Eur. J. Biochem. 196*, 499–507.
9. Rustan, A.C., Christiansen, E.N., and Drevon, C.A. (1992) *Biochem. J. 283*, 333–339.
10. Halminski, M.A., Marsh, J.B., and Harrison, E.H. (1991) *J. Nutr. 121*, 1554–1561.
11. Yamazaki, R.K., Shen, T., and Schade, G.B. (1987) *Biochim. Biophys. Acta 920*, 62–67.
12. Nossen, J. Ø., Rustan, A.C., Gloppestad, S.H., Målbakken, S., and Drevon, C.A. (1986) *Biochim. Biophys. Acta 879*, 56–65.
13. Otto, D.A., Baltzell, J.K., and Wooten, J.T. (1992) *Lipids 27*, 1013–1017.
14. Phillipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S., and Illingworth, D.R. (1985) *N. Eng. J. Med. 312*, 1210–1216.
15. Igarashi, O. (1993) *Vitamin E—Its Usefulness in Health and in Curing Diseases* (Mino, M., Nakamura, H., Diplock, A.T., and Kayden, H.J., eds.) pp. 131–142, Japan Scientific Societies Press, Tokyo.
16. Folch. J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
17. Zak, B. (1957) *Am. J. Clin. Path. 27*, 583–588.
18. Van Handel, E. (1961) *Clin. Chem. 7*, 249–251.
19. Chen, P.S., Toribara, T.Y., and Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
20. Yagasaki, K., Okada, K., Takagi, K., and Irikura, T. (1984) *Agric. Biol. Chem. 48*, 1417–1423.
21. Kushiro, H., Takano, Y., Soyama, K., and Fukui, I. (1970) *Japan. J. Clin. Path. 18*, 51–54.
22. Noguchi, Y., Vydelingum, N.A., Younes, R.N., Fried, S.K., and Brennan, M.F. (1991) *Cancer Res. 51*, 863–869.
23. Nilsson-Ehle, P., and Schotz, M.C. (1976) *J. Lipid Res. 17*, 536–541.
24. Nakai, T., Yamada, S., Tamai, T., Kobayashi, T., Hayashi, T., and Takeda, R. (1979) *Metabolism 28*, 30–40.
25. Fredrikson, G., Strålfors, P., Nilsson, N.Ö., and Belfrage, P. (1981) *J. Biol. Chem. 256*, 6311–6320.
26. Yagasaki, K., Okada, K., Mochizuki, T., Takagi, K., and Irikura, T. (1984) *Biochem. Pharmac. 33*, 3151–3163.
27. Okada, K., Yagasaki, K., Mochizuki, T., Takagi, K., and Irikura, T. (1985) *Biochem. Pharmac. 34*, 3361–3367.
28. Yamanaka, Y., Tsuji. K., and Ichikawa, T. (1986) *J. Nutr. Sci. Vitaminol. 32*, 287–296.
29. Sautier, C., Dieng, K., Flament, C., Doucet, C., Suquet, J.P., and Lemonnier, D. (1983) *Br. J. Nutr. 49*, 313–319.
30. Yagasaki, K., Machida-Takehana, M., and Funabiki, R. (1990) *J. Nutr. Sci. Vitaminol. 36*, 45–54.
31. Duncan, D.B. (1955) *Biometrics 11*, 1–42.
32. Yagasaki, K. (1989) *Absorption and Utilization of Amino Acids* (Friedman, M., ed.) Vol. II, pp. 275–287, CRC Press, Florida.
33. Ikeda, I., Nakashima-Yoshida, K., and Sugano, M. (1985) *J. Nutr. Sci. Vitaminol. 31*, 375–384.
34. Thompson, M.P., Cooper, S.T., Parry, B.R., and Tuckey, J.A. (1993) *Biochim. Biophys. Acta 1180*, 236–242.
35. Damen, J., Van Ramshorst, J., Van Hoeven, R.P., and Van Blitterswijk, W.J. (1984) *Biochim. Biophys. Acta 793*, 287–296.

[Received September 14, 1994, and in revised form February 20, 1995; Revision accepted March 20, 1995]

Dietary Fish Oils and Long-Term Malaria Protection in Mice

Per Fevang[a,b], Hjalmar Sääv[b] and Arne T. Høstmark[c]

[a]Center for Tropical Parasitic Diseases, Department of Microbiology and Immunology, Miami, Florida, [b]Pronova Biocare, Sandefjord, Norway and [c]Department of Preventive Medicine, University of Oslo, Norway

ABSTRACT: Previous studies indicate a suppressive influence of fish oils on rodent malaria. The present work was carried out to study (i) the dose–effect relation between dietary fish oils and lethality of primary malaria infection in mice; (ii) the modifying influence of vitamin E; and (iii) the effect of previous fish oil feeding on parasitemia and lethality of a rechallenge infection. For two or four weeks, groups of weanling male mice were fed a standard laboratory diet or one of eight purified diets containing various amounts of fish oil (providing 6–21% of energy). The diets were prepared with and without vitamin E. After the two- or four-week feeding period, the mice were injected intraperitoneally with *Plasmodium yoelii yoelii*-infected erythrocytes. Six months after the primary infection (four months after discontinuing fish oil feeding), the surviving mice were again injected intraperitoneally with parasitized red blood cells (or even better—erythrocytes, erythrocytes are used elsewhere). Primary malaria infection was lethal in mice fed standard diet alone or with fish oil and vitamin E added. In contrast, feeding a fish oil-based diet without vitamin E improved survival to at least 70% if the mice had been fed these diets for four weeks. Protection against malaria did not seem to be related to the fish oil dose used. Regardless of the previous fish oil dose, all the mice surviving the primary infection survived the rechallenge infection with low parasitaemias. The results suggest that the prooxidant nature of highly unsaturated fatty acids in fish oils may beneficially influence malaria infection, and may also increase the resistance against reinfection for some time after discontinuing fish oil intake.

Lipids 30, 437–441 (1995).

Malaria is a serious global threat to human health. The need for effective forms of treatment and prophylaxis against the disease is of great interest. The situation is worsening with increasing parasite and mosquito vector adaptation to drugs and insecticides (1). The suppressive action of a vitamin E-deficient n–3 fatty acid diet on plasmodium infection in mice has been known since 1957 (2–4). Vitamin E reversed the suppressive action of fish oils, suggesting increased lipid peroxidation as a mechanism behind the effect. Cream rich in saturated fatty acids, and olive oil rich in monounsaturated fatty acids had no effect on survival or on parasitemia.The protection against malaria rechallenge by a 5% menhaden oil (MO)-enriched vitamin E-deficient diet was reported in abstract form by Ager *et al.* (5). The authors observed that once mice were cured of malaria by feeding this prooxidant diet, the animals were largely protected against malarial rechallenge.

The long-chain polyunsaturated n-3 fatty acids (PUFAs), e.g., eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are important components of the phospholipids in cell membranes. Intake of fish oil rich in n-3 fatty acids results in increased amounts of these fatty acids in plasma and in cell membranes. When exposed to oxygen, PUFAs easily give away one hydrogen atom, thereby forming free radicals which may start a free radical chain reaction. Vitamin E in cell membranes stops the peroxidation of long- chain highly unsaturated fatty acids (6).

The purpose of the present work was to study (i) the dose–effect relation between dietary fish oils and lethality of primary malaria infection in mice; ii) the modifying influence of vitamin E; and iii) the effect of previous fish oil feeding on parasitemia and lethality of a rechallenge infection.

MATERIALS AND METHODS

Groups of 15 weanling, three-week-old male outbred CD-1 Swiss albino mice were fed one of eight purified diets (Table 1) or a Purina Chow (Formulab Chow 5008^{R}, Ralston Purina Co., St. Louis, MO) for two or four weeks. The fat in the purified diets was either MO 4 g/100 g plus corn oil 1 g/100 g [diet MO], or the diets containing different amounts of EPAX fish oil concentrate (2.4 or 8 g/100 g) plus corn oil (0.5,1.0, or 2.0 g/100 g). MO contained 9.1% w/w DHA and 11.0% EPA, obtained from National Marine Fisheries service (U.S. Department of Commerce, Charleston, SC). The fish oil triglyceride EPAX 3000 contained 11.4% DHA and 17.8% EPA, and was from Pronova Biocare A/S (Sandefjord, Nor-

*To whom correspondence should be addressed at Pronova Biocare, P.O. Box 2109, 3201 Sandefjord, Norway.

Abbreviations: DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); MO, menhaden oil; PUFA, polyunsaturated fatty acid.

Copyright © 1995 by AOCS Press

TABLE 1
Diet Components (as g/100 g of diets)

Diet	Casein	Salts[a]	Vitamins[b]	Vitamin E[c]	MO[d]	Corn oil[e]	EPAX[f]	DL-m[g]	Choline[h]	Sucrose
MO + VE	20	3.5	1	0.00385	4	1	0	0.3	0.225	69.97
MO – VE	20	3.5	1	0	4	1	0	0.3	0.225	69.98
2% EPAX + VE	20	3.5	1	0.00385	0	0.5	2	0.3	0.225	72.47
2% EPAX – VE	20	3.5	1	0	0	0.5	2	0.3	0.225	72.48
4% EPAX + VE	20	3.5	1	0.00385	0	1	4	0.3	0.225	69.97
4% EPAX – VE	20	3.5	1	0	0	1	4	0.3	0.225	69.98
8% EPAX + VE	20	3.5	1	0.00385	0	2	8	0.3	0.225	64.97
8% EPAX – VE	20	3.5	1	0	0	2	8	0.3	0.225	64.98

[a]AIN-76 salt mix without selenium, diet supplemented with 0.1 mg selenium per kg as sodium selenite.
[b]AIN-76A vitamin mix without vitamin E (VE) diet also contained 20 mg para-aminobenzoic acid per kg.
[c]Added as d-alpha-tocopheryl acetate (equivalent to 0.0035 g of d-alpha-tocopherol).
[d]MO (menhaden oil) contained 11.0% eicosapentaenoic acid (EPA) and 9.1% docosahexaenoic acid (DHA) as triglyceride, supplied by National Marine Fisheries Service, U.S. Department of Commerce (Charleston, SC).
[e]Stripped of vitamin E.
[f]EPAX contained 11.43% EPA and 17.81% DHA as triglyceride, supplied by Pronova A/S (Sandefjord, Norway).
[g]DL-methionine.
[h]Added as choline dihydrogen citrate.

way). The purified diets were prepared with and without d-alpha tocopherylacetate, referred to as vitamin E, to obtain eight purified diets (Table 1). The antioxidant tertiary butylhydroquinone (200 ppm) was added to the diets to prevent lipid peroxidation, and the diets were stored at 4°C.

Protein, carbohydrate, and fat provided 20, 69, and 11% of energy in the MO and the 4% EPAX diets. Corresponding energy distribution in the 2% EPAX diets was 20, 74, and 6 and 19, 60, and 21% in the 8% EPAX diets. Energy densities (MJ/100 g) were 1.70 (MO and 4% EPAX diets), 1.65 (2% EPAX diets), and 1.81 (8% EPAX diets). The mice were kept in metal-topped plastic cages and had free access to their diets and drinking water. The room housing the mice was maintained at 24°C and 66% relative humidity with a 12-h light/dark cycle. Mice were maintained according to the National Research Council's guide for the care and use of laboratory animals at the Center for Tropical Parasitic Diseases, Department of Microbiology and Immunology, University of Miami, (Miami, FL).

After two or four weeks, ten of the mice in each group were injected intraperitoneally with 5×10^4 red blood cells parasitized with *Plasmodium yoelii yoelii* (primary infection study). At the same time five of the mice in each group were killed, and plasma was saved for vitamin E analyses. Plasma vitamin E concentrations were analyzed by high-performance liquid chromatography using the procedure of Bieri *et al.* (7).

Previous experience has demonstrated that 60 days postinfection is sufficient to judge the parasitemia and survival of infected mice (3). At that time, samples of tail blood were examined for parasites with a light microscope by the following procedure: we examined 100 erythrocytes if parasitemia was 10–100%, 200 erythrocytes if parasitemia was 5–10%, 2,000 erythrocytes if parasitemia was 0.5–5% (until ten parasites were found), 2,000–20,000 erythrocytes if parasitemia was 0.01–0.45% (until one parasite was found), and 20,000 erythrocytes without a parasite before the mouse was considered free of parasites.

The mice were maintained on the fish oil diets for another eight weeks; then the fish oil supplementation was stopped and the diet changed to a standard Chow diet for four months (Formulab Chow 5008^R, Ralston Purina Co.) Still on the standard Chow diet, the mice surviving after the primary infection were again reinjected intraperitoneally with 5×10^4 red blood cells parasitized with *P. yoelii yoelii* (Rechallenge trial). The chloroquine sensitive *P. yoelii yoelii* was originally obtained from Dr. Richard Jacobs in 1973 and has been routinely passed through *Anopheles stephensi* in the laboratory of Dr. Arba L. Ager (Miami, FL) to maintain viable gametocytes.The origin of this CS line is described in detail by Rane and Kinnamon (8).

Statistics. Biochemical and parasitemia data were analyzed by analysis of variance and Duncan's multiple range test (9). The survival data were analyzed by the Wilcoxon rank test (10). For analysis of vitamin E data, we used Student's *t*-test to estimate the difference between means.

RESULTS

Primary infection. To evaluate the dose–effect of fish oils on the survival rate following a primary infection with malaria, mice were pre-fed Chow or vitamin E-deficient diets with various concentrations of fish oils. Primary malaria infection was lethal in mice fed Chow or fish oil diets with vitamin E (Table 2). In contrast, feeding fish oil minus vitamin E improved survival to at least 70% in all mice fed fish oil diets for four weeks. Increasing the fish oil dose above 2% did not seem to increase protection. A shorter pre-feeding interval (two weeks) was generally less effective than four weeks of prefeeding, but the global difference was not statisistically significant. Table 3 shows the serum vitamin E concentration after the primary feeding period. Groups fed vitamin E-deficient fish oil diets had appreciably lower values ($P < 0.05$) than groups fed Chow, or fish oil diets supplemented with vitamin E.

Rechallenge survival and parasitemia. Prefeeding Chow

TABLE 2
Total Sixty-Day Survival of Mice in Malaria Primary Infection[a]

Diet	Vitamin E (% of diet)	Two weeks prefeeding (survivors/total)	Four weeks prefeeding (survivors/total)
Chow		0/10	0/10
4% Menhaden oil[b]	0	4/10	8/10
4% Menhaden oil	0.00384	0/10	0/10
2% EPAX[c]	0	8/10	8/10
2% EPAX	0.00384	0/10	0/10
4% EPAX[c]	0	7/10	9/10
4% EPAX	0.00384	0/10	0/10
8% EPAX[c]	0	5/10	7/10
8 % EPAX	0.00384	0/10	0/10

[a]Mice were prefed various diets as described in the Materials and Methods section. The animals were then injected intraperitoneally with *Plasmodium yoelii yoelii* infected erythrocytes. Numbers of surviving animals/total number mice are shown. Abbreviations as in Table 1.
[b]Menhaden oil contained 11% EPA and 9% DHA.
[c]EPAX contained 18% EPA and 12% DHA.

TABLE 3
Plasma Vitamin E in Primary Challenge Study

Diet	Vitamin E[a]	Two weeks prefeeding (mol/l)	Four weeks prefeeding (mol/l)
Chow		5.1 ± 0.4	5.6 ± 0.7
4 % MOb	–	1.4 ± 0.2	0.9 ± 0.1[e]
4 % MO	+	5.0 ± 0.4[d]	4.8 ± 0.6[d]
2 % EPAXc	–	1.1 ± 0.1	
2 % EPAX	+	4.9 ± 0.6[d]	
4 % EPAX	–	1.2 ± 0.1	0.9 ± 0.2
4 % EPAX	+	4.4 ± 0.2[d]	6.8 ± 0.1[d,e]
8 % EPAX	–	1.0 ± 0.1	1.1 ± 0.2
8 % EPAX	+	4.5 ± 0.7[d]	5.6 ± 0.6[d]

[a]d-Alpha-tocopherylacetate 0.00385 g/100 g diet.
[b]Menhaden oil (MO) contained 11% EPA and 9% DHA. Abbreviations as in Table 1.
[c]EPAX contained 18% EPA and 12% DHA.
[d]$P < 0.05$ vs. corresponding group without vitamin E (by unpaired Student's *t*-test, mean values ± SEM are shown).
[e]$P < 0.05$ vs. two weeks prefeeding (by unpaired Student's *t*-test).

TABLE 4
Rechallenge Survival and Parasitemia[a]

	Diet during primary infection	Mice surviving rechallenge (survivors/total)	Six-day parasitemia (% infected erythrocytes)
Two weeks prefeeding	4% Menhaden oil[b]	3/3	0.4 ± 0.3
	2% EPAX[c]	8/8	0.4 ± 0.2
	4% EPAX[c]	7/7	0.1 ± 0.0
	8% EPAX[c]	5/5	0.1 ± 0.0
	Control	0/10	45 ± 6
Four weeks prefeeding	4% Menhaden oil[b]	8/8	0.1 ± 0.1
	2% EPAX[c]	8/8	0.1 ± 0.1
	4% EPAX[c]	9/9	0.3 ± 0.2
	8% EPAX[c]	7/7	0.2 ± 0.1
	Control	0/10	45 ± 6

[a]Mice surviving the primary infection reported in Table 3 were fed fish oil diets (see Materials and Methods section) for two months, and then Chow diet for four months. The animals were then again injected intraperitoneally with *Plasmodium yoelii yoelii*-infected erythrocytes. Control mice were fed Chow.
[b]Menhaden oil contained 11% EPA and 9% DHA.
[c]EPAX contained 18% EPA and 12% DHA.

offered no protection against malaria (Table 4). In contrast, all the surviving animals from the primary infection survived a malaria parasite rechallenge after six months. Whereas Chow prefeeding resulted in a high parasitemia, all the fish oil groups had low parasitemias of 0.1–0.4%. All of the mice that had, previously received fish oil were free of parasites by day 13 after infection. The survival and parasitemia in the rechallenge infection seemed to be independent of the dose of fish oil fed during the primary infection.

DISCUSSION

This study confirms a preliminary report of the protective effect of vitamin E-deficient, fish oil-enriched diets on parasitemia and survival during reinfection after a primary *P. yoelii yoelii* infection in mice (5). In addition, the present work demonstrates that the antimalarial effect of fish oil can persist even four months after stopping the fish oil diet, and that the effect was not clearly dose-related in the range studied (i.e., 2–8%). The present results do not, however, elucidate the mechanisms involved in the persistent malaria-protective influence of fish oils.

The lipid composition of the cell membranes reflects dietary fatty acids (11). Moreover, cell membranes of the malaria parasite are greatly influenced by the diet of the host. Conceivably, membranes formed with a surplus of PUFA and a lack of vitamin E may be more susceptible to lipid peroxidation than cell membranes formed under conditions with less PUFA available and more vitamin E. In this context, PUFA of fish oil are especially peroxidation-prone in the absence of vitamin E. It would appear, therefore, that lipid peroxidation is at least partly involved in the observed effects. This suggestion is supported by the fact that vitamin E abolished the protective influence of fish oils in the primary infection trial. Thus, the protective effect of fish oils apparently is not caused by the intact fatty acids of fish oil *per se*. Whether vitamin E supplementation can also modify a rechallenge infection was not investigated in this study.

The course of the malaria infection requires a rapid regeneration of parasites with a high degree of plasmodial membrane biosynthesis. It was demonstrated in mice at a late stage of infection that the phospholipid content of *Plasmodium vinckeii* parasitized mouse erythrocytes was elevated more than fourfold compared to uninfected cells, with a relative increase in the ratio of unsaturated-to-saturated fatty acids in the phospholipids (12). Most of this increase could be attrib-

uted to the presence of parasite membranes. There was also a decrease in the vitamin E content of the infected erythrocytes that would act synergistically to increase the parasite membrane susceptibility to peroxidative damage.

In vitro studies have demonstrated that increasing concentrations of DHA have an increasing parasitolytic effect on a Chloroquine-sensitive Tanzanian parasite strain (F 32) of *Plasmodium falciparum* (13). Full growth inhibition was obtained with 0.05 mM or higher DHA concentrations in the medium. *Plasmodium falciparum* is the parasite that causes the most lethal type of malaria in humans.

The strong inhibition of malaria parasite multiplication by fish oil, or *in vitro* by pure ν–3 fatty acids, may have some similarities to the inhibiting effect of PUFA on the growth of human hepatoma cells (14,15). In these latter studies, it was suggested that the growth inhibitory effect depended on lipid peroxidation as there was a parallel formation of thiobarbituric acid reactive substances, an index of PUFA peroxidation. Furthermore, PUFA-induced growth inhibition was strongly antagonized by the antioxidant vitamin E. Also, increasing the albumin concentration prevented growth inhibition by PUFA and the appearance of thiobarbituric acid reactive substances. Although it is well known that albumin is a carrier of fatty acids, its role to protect against growth inhibition by PUFAs may also relate to its extracellular antioxidant effects (16).

Related to the hypothesis that lipid oxidation *in vivo* can affect the clinical expression of malaria are the findings that grossly undernourished nomads from sub-Saharan districts developed severe malaria when they were refed with relief grain and cereals which are high in vitamin E and protein, i.e., making a favorable situation for the malaria parasite (17). Moreover, refeeding with grain exacerbated latent malaria in undernourished adults and precipitated severe cerebral malaria in young undernourished children (18). As one would hope that refeeding would eventually increase the resistance against infectious and parasitic diseases, some of these unexpected findings could be attributed to the antioxidant influence of vitamin E protecting the parasites or to increased ability to carry on protein synthesis by the parasites, brought about by feeding relief grain with its several nutrients. Interestingly, infants from Papua New Guinea with riboflavin deficiency had lower malaria parasite densities than infants with normal riboflavin status (19). This finding is consistent with results from rodent malaria studies (20), and with the idea that both parasite and host nutrition can be closely interrelated.

The severity and duration of malaria attacks depend on many factors, including the nutritional status of the host and the virulence of the infective strain. The most important modifying factor, however, is the development of resistance in the infected individual. It is well known that a child surviving to the age of 5–6 years in areas with very high transmission of *P. falciparum* will have achieved a high degree of resistance to the lethal effects of the infection. Specific malaria antibodies have been demonstrated in *P. yoelii yoelii* infections in rodents (21). Similar findings have been made in simian and in human malaria. It remains to be established, however, to what extent immunological resistance was part of the protection against the rechallenge infection observed in the present study. Our study demonstrates that the malaria protection in the rechallenge infection after four months on a standard laboratory diet is independent of dietary fish oil concentration in the primary feeding period if the dose is above 2%. It seems likely that the erythrocyte concentration of n-3 fatty acids would be very low after four months without fish oil in the diets (11). In contrast, standard antimalarial therapy is associated with substantially depressed antibody levels (22), and the widespread use of antimalarials has not been associated with protection against rechallenge infection.

The present diet study in mice supports the contention that there is a connection between the severity of a malaria infection and the state of nutrition. It would appear that a nontoxic nutritional approach with n-3 fatty acid supplements could beneficially influence the course of malaria infection and increase the resistance against a reinfection. Further research is necessary to support this suggestion and to determine the mechanism of this effect of fish oil.

ACKNOWLEDGMENTS

The experimental work of the study was performed at the Center of Tropical Parasitic Diseases (Miami, FL) under the guidance of Dr. Arba L. Ager. We also thank Dr. Orville A. Levander, Vitamin and Mineral Nutrition Laboratory (Beltsville, MD), for scientific support with the planning and performance of the study. This work was supported by Pronova Biocare A/S (Sandefjord, Norway).

REFERENCES

1. WHO Expert Committee (1985) *Lancet 2*, 791.
2. Godfrey, D.G. (1957) *Exp. Parasitol. 6*, 555–565.
3. Levander, O.A., Ager, A.L., Morris, V.C., and May, R.G. (1989) *Am. J. Clin. Nutr. 50*, 346–352.
4. Kumaratilake, L.M., Robinson, B.S., Ferrante, A., and Poulos, A. (1992) *J. Clin. Invest. 89*, 961–967.
5. Ager, A.L., Levander, O.A., Morris, R., May, F., and May, R.G. (1992) *FASEB J. 6*, A1212.
6. Vatassery, G.T. (1989) *Lipids 24*, 299–304.
7. Bieri, J.G., Tolliver, T.J., and Catignani, G.L. (1979) *Am. J. Clin. Nutr. 32*, 2143–2149.
8. Rane, D.S., and Kinnamon, K.E. (1979) *Am. J. Trop. Med. Hyg. 28*, 937–947.
9. Steel, R.G.D., and Torrie, J.H. (1960) *Principles and Procedures of Statistics*, McGraw-Hill, New York.
10. Lee, E.T. (1980) *Statistical Methods for Survival Data Analysis*, Lifeline Learning Publications, Belmont.
11. Flaten, H., Høstmark, A.T., Kierulf, P., Lystad, E., Trygg, K., Bjerkedal, T., and Osland, A. (1990) *Am. J. Clin. Nutr. 52*, 300–306.
12. Stocker, R., Cowden, W.B., Tellam, R.L., Weidemann, M.J., and Hunt, N.H (1987) *Lipids 122*, 51–57.
13. Fevang, P., Høstmark, A.T., and Bjørkmann, A. (1992) *XIIth International Congress for Tropical Medicine and Malaria*, Jomtien, Pattaya, Thailand Abstr. 2,268.18.
14. Høstmark, A.T., and Lystad, E. (1992) *Acta Physiol. Scand. 144*, 83–88.
15. Lystad, E., Høstmark, A.T., Kiserud, C., and Haugen, A.A (1994) *In Vitro Cell. Dev. Biol. 30A*, 568–573.

16. Halliwell, B. (1988) *Biochem. Pharm. 37*, 569–571.
17. Eckman, J.R., Eaton, J.W., Berger, E., and Jacob, H.S. (1976) *Trans. Assoc. Am. Phys. 89*, 105–113.
18. Murray, M.J., and Murray, A.B. (1977) *Persp. Biol. Med. 20*, 471–483.
19. Thurnham, D.I., Oppenheimer, S.J., and Bull, R. (1983) *Trans. Royal. Soc. Trop. Med. Hyg. 77*, 423–424.
20. Kaikai, P., and Thurnham, D.I. (1983) *Trans. Royal. Soc. Trop. Med. Hyg. 77*, 680–686.
21. Wernsdorfer, W.H., and McGregor, I. (1988) *Malaria, Principles and Practice of Malariology*, p. 516, Churchill Livingstone, Edinburgh.
22. Wernsdorfer, W.H., and McGregor, I. (1988) *Malaria, Principles and Practice of Malariology*, p. 590, Churchill Livingstone, Edinburgh.

[Received June 15, 1994, and in revised form March 13, 1995; Revision accepted March 17, 1995]

Dietary Deficiency of Docosahexaenoic Acid Impairs Vision at Low Light Intensities in Juvenile Herring (*Clupea harengus* L.)

Michael V. Bell[a,*], Robert S. Batty[b], James R. Dick[a], Karen Fretwell[b,1], Juan Carlos Navarro[a,2] and John R. Sargent[a]

[a]Natural Environment Research Council, Unit of Aquatic Biochemistry, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, United Kingdom and [b]Natural Environment Research Council, Dunstaffnage Marine Laboratory, Oban, Argyll PA34 4AD, United Kingdom.

ABSTRACT: In the retina of herring (*Clupea harengus* L.), rods are recruited from about 8 wk after hatching, and from this time there is a linear relationship between the number of rods in the photoreceptor cell population and the content of di22:6n-3 molecular species of phospholipids. Juvenile herring were reared from four weeks' post-hatching for 15 wk on either *Artemia* nauplii deficient in 22:6n-3 or on enriched *Artemia* nauplii containing 4.3% 22:6n-3. The visual performance of the fish was then determined at three light intensities (0.01, 0.1, and 1.0 lux) by observing their frequency of striking at live *Artemia* nauplii using infrared video recording. Herring reared on the diet containing no 22:6n-3 were less active predators, especially at the lowest light intensity where very few strikes were observed. The eyes of these fish contained greatly reduced levels of di22:6n-3 molecular species of total phospholipid, 2.1% vs. 12.0% in fish supplemented with 22:6n-3. The contribution of saturated and monounsaturated fatty acids in the molecular species of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) was virtually unchanged, while 20:5n-3 and 22:5n-3 largely replaced 22:6n-3. There was an almost complete disappearance of di22:6n-3 PC, while the amounts of di22:6n-3 PE and PS fell by 18.1 and 20.6% to 2.7 and 7.6%, respectively. The dipolyunsaturated molecular species di20:5n-3, 20:5n-3/22:5n-3, and di22:5n-3 made up a substantial part of the deficit. We conclude that a dietary deficiency of 22:6n-3 during the period early in rod development impairs visual performance such that the fish can no longer feed at low light intensities.

Lipids 30, 443–449 (1995).

In terrestrial vertebrates, docosahexaenoic acid (22:6n-3) is concentrated in neural tissue, especially in retina and brain (1,2) and testes (3), and this is the case also in marine vertebrates, including fish, even though fish tissues in general are richer in 22:6n-3 than mammalian tissues. Vertebrate retinae contain large amounts of di-docosahexaenoyl (22:6n-3/22:6n-3) glycerophospholipid which is concentrated in rod outer segment membranes (1,4,5). However, the concentration of 22:6n-3 in the retina of fish is particularly impressive, e.g., in cod (*Gadus morhua*) 71.8% of phosphatidylethanolamine (PE), 59.7% of phosphatidylserine (PS), and 29.3% of phosphatidylcholine (PC) occurs as 22:6n-3/22:6n-3 molecular species (6). The amounts in the retina of herring are slightly lower, equivalent to 30.6% of total glycerophospholipid in adult fish (7). Therefore, there is strong circumstantial evidence that 22:6n-3 is essential for neural function.

Docosahexaenoic acid (22:6n-3) is an essential fatty acid in carnivorous species of fish and other animals which appear to have lost the Δ5 desaturase and cannot therefore elongate and desaturate dietary precursor C_{18} polyunsaturated fatty acids (PUFA) to C_{20} PUFA (8–10). It is now believed that the final step in the formation of 22:6n-3 in mammals involves Δ6 desaturation of 24:5n-3 and subsequent chain-shortening of 24:6n-3 (11), and the same pathway probably operates in fish. Furthermore, in some species the ability to synthesize 22:6n-3 from dietary precursors at times of high demand during neural development may be inadequate, and 22:6n-3 deficiency at this time causes impairment of visual and cognitive abilities in young animals (12,13). Recent evidence suggests this is also the case in human infants, especially preterm infants, and that such deficiencies lead to visual and cognitive subperformance (14–16). This has led to the recommendation in the United Kingdom that infant formula feeds should contain 22:6n-3 (17).

Induction of 22:6n-3 deficiency in experimental animals is difficult since 22:6n-3 is retained avidly in neural tissues (12,13), and experiments to test visual and behavioral subper-

*To whom correspondence should be addressed.
[1]Deceased.
[2]Present address: Instituto de Acuicultura de Torre la Sal, 12595 Torre de la Sal, Castellon, Spain.

Abbreviations: ANOVA, analysis of variance; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. Molecular species are abbreviated as follows: e.g., 16:0/22:6 PC is 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

Copyright © 1995 by AOCS Press

formance are difficult to devise, especially with a view to separating effects on vision (performance of retina) and cognition (performance of brain) (18). The free-swimming larvae of marine fish offer a number of advantages over fetal mammals for studies on the effects of 22:6n-3 deficiency. Their small size and rapid growth from first-feeding mean that they have a very large demand for 22:6n-3 which must be met directly from the diet, and deficiencies of 22:6n-3 are thus easy to induce. At hatching, the larvae of many species of marine fish have almost pure cone retinae with rods appearing later in development. In herring larvae, significant numbers of rods are recruited to the photoreceptor cell population progressively from about 8 wk after hatching (length 2.5 cm) to maturity (length 25–30 cm) to give a rod/cone ratio of 15–20:1 (19). The length of the fish is a good indicator of rod development (19). It has been shown that the amount of di22:6n-3 PE, PS, and PC in herring eyes correlates with the proportion of rods in the photoreceptor population and that the pure cone retina of larvae contains about half the amount of di22:6n-3 phospholipid in adult retina (7). The present study sought to reduce the content of di22:6n-3 phospholipid in the eyes of young herring by feeding a diet lacking 22:6n-3 and to determine whether this affected the visual performance of the fish as measured by their ability to detect and strike at prey at different light intensities chosen specifically to test cone vision and rod (scotopic) vision.

MATERIALS AND METHODS

Experimental animals. Spring spawning herring were caught in the Firth of Clyde, Scotland. The mixed progeny from artificially fertilized eggs from twelve females and six males were reared in 450-L tanks containing seawater with a salinity of 30 to 32‰. Ambient seawater temperatures were used and rose from 8.5 ± 0.4°C during the embryo stage to 13.1 ± 0.7°C during the last week of the experiment. Larvae were fed initially from eight days' post-hatching on nauplii of *Artemia* sp. (AF grade; Artemia Systems, Baasrode, Belgium). After 20 days' feeding on this diet, larvae were transferred to the experimental diets which consisted of two strains of San Francisco Bay *Artemia* nauplii with relatively high or low n-3 fatty acid contents, the former supplemented with n-3 PUFA to provide 4.3% of fatty acids as 22:6n-3 and the latter supplemented with coconut oil giving no 22:6n-3 (Table 1, see Ref. 20 for details). Two replicate tanks of larvae were fed on each of these diets for a further 102 d before sampling.

Chemicals and solvents. Phospholipase C from *Bacillus cereus* was purchased from Boehringer Corporation (London) Ltd. (Lewes, East Sussex, England). Oxalyl chloride and anthracene-9-carboxylic acid were supplied by Aldrich Chemical Co. (Gillingham, Dorset, England). All other chemicals and biochemicals were purchased from Sigma (Poole, Dorset, England), and solvents of high-performance liquid chromatography (HPLC) grade were obtained from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Measurement of visual performance. Twenty fish were sampled at random from each tank, and their feeding behavior recorded in tanks 59 cm in diameter using an infrared video recording system with strobed infrared illumination (21). Infrared illumination was provided by a tungsten halogen bulb and infrared transmitting filter with a sharp cut-off at 700 nm which is outside the reported range of spectral sensitivity for larval and adult herring (22,23) or of any other marine fish species (24). Diffuse visible light was provided by a tungsten halogen lamp through a fiber-optic light guide fitted with neutral density filters and reflected off a white background 2 m above the tanks. The light intensity was measured at the water surface with an EEL micro photometer (Evans Electroselenium Ltd., Halstead, Essex, United Kingdom). Three replicate five-minute experiments were performed with groups of 20 fish from each tank. Three visible light intensities of 0.01, 0.1, and 1 photopic lux (equivalent to 50, 500, and 5000 $\mu W \cdot m^{-2}$) were used with different randomly chosen groups of 20 fish. In order to ensure complete adaptation to the low light intensities used, the fish were first dark-adapted by keeping them in complete darkness overnight. They were not fed during this time (approximately 16 h), and their guts were empty at the start of the experiment. They were then adapted to the experimental light intensity for 30 min before introducing prey (*Artemia* sp. nauplii at a density of 1000 L^{-1}) and commencing video recording. The larvae were returned to their rearing tanks after the behavior experiments. Subsequent frame-by-frame analysis of the video recordings (20 ms intervals) revealed the total number of strikes made at prey per fish per minute. The single camera recording system allowed analysis in only two dimensions, and therefore successful and unsuccessful feeding strikes could not be distinguished reliably.

Lipid extraction and analysis. Twenty herring larvae were anaesthetized in 0.02% (wt/vol) ethyl 3-aminobenzoate methane sulphonate (MS222), their lengths measured, and their eyes removed. The eyes and carcases were immediately frozen in liquid nitrogen and subsequently stored at −70°C. Total lipids were extracted from the eyes of individual fish by sonication in 2 mL of chloroform/methanol 2:1 (vol/vol) containing 0.01% (wt/vol) butylated hydroxytoluene as antioxidant and a Folch extract prepared as previously described (25). Samples were stored at −20°C under nitrogen between

TABLE 1
The Fatty Acid Composition of the Experimental Diets (wt%)[a]

Fatty acid	22:6n-3-Supplemented	22:6n-3-Deficient
18:2n-6	3.9	6.3
18:3n-3	12.2	20.6
20:5n-3	11.6	2.9
22:5n-3	0.8	0
22:6n-3	4.3	0
Total saturates	16.4	22.7
Total monounsaturates	33.8	31.1
Total n-3 PUFA	32.6	27.4
Total n-6 PUFA	4.1	6.3

[a]See Reference 20 for full details. PUFA, polyunsaturated fatty acid.

preparative procedures. The fatty acid composition of total eye lipid of five fish matched for size from each tank was determined by gas–liquid chromatography of fatty acid methyl esters using a Packard 436 gas chromatograph fitted with a fused silica capillary column (50 m x 0.25 mm) coated with FFAP phase (S.G.E., Milton Keynes, United Kingdom) using hydrogen as carrier gas (26).

Molecular species analysis. To determine the di22:6n-3 content of total phospholipid, neutral lipid was removed by thin-layer chromatography (TLC) on high-performance TLC (HPTLC) silica gel 60 plates (Merck, Darmstadt, Germany) in hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) and the phospholipid remaining on the origin scraped from the HPTLC plate. The glycerophospholipid was hydrolyzed with phospholipase C (*B. cereus*) [Boehringer Corporation (London) Ltd.] and the 9-anthroyl derivatives prepared as described previously (7,27). The eyes from ten fish matched for size from each tank were analyzed.

PC, PE, and PS were prepared from 100 μg of total lipid by HPTLC in methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aq. KCl (25:25:25:10:9, by vol) (28). The edges of the bands were visualized with iodine, and the remaining sample scraped from the plate before hydrolyzing with phospholipase C as before. The eyes from two fish matched for size (4.0 cm) from each tank were analyzed. Molecular species were separated by HPLC on an Ultrasphere C18 column (25 x 0.46 cm, 5 μm particle size) (Beckman Instruments U.K. Ltd., High Wycombe, United Kingdom) using isocratic elution with acetoni-trile/propan-2-ol, 7:3 (vol/vol) at a flow rate of 1.0 mL/min and methanol/propan-2-ol, 4:1 (vol/vol) at a flow rate of 1.0 mL/min to separate any unresolved peaks. Peaks were detected using a Waters (Watford, United Kingdom) 470 scanning fluorescence detector (excitation 360 nm, emission 460 nm) and quantified with a Shimadzu CR3A recording integrator (Tokyo, Japan). Peaks were identified with reference to standard lipids and samples of known composition (6,7,29).

Data analysis. The behavioral data and the total di22:6n-3 phospholipid content were analyzed with a SAS statistical package by repeated measures multivariate two-way analysis of variance (ANOVA). The molecular species composition of individual lipid classes was analyzed by one-way ANOVA.

RESULTS

At the end of the experimental period, there was no significant difference ($P = 0.3366$) in the size of fish fed the two diets or those used in the behavior experiments. Lengths ranged from 2.5–4.9 cm with mean sizes in the replicate tanks of 3.48 ± 0.60 cm and 3.68 ± 0.41 cm for the 22:6n-3-supplemented fish and 3.68 ± 0.37 cm and 3.87 ± 0.58 cm for the 22:6n-3-deficient fish.

The threshold light intensity for visual feeding by dark-adapted adult herring is 0.01 lux (30). At this light intensity, there was a significant difference in the behavior of the fish reared on the two dietary regimes (ANOVA, $P = 0.0044$). Fish reared on the 22:6n-3-supplemented diet fed well, but almost none of the fish reared on the diet lacking 22:6n-3 attacked the prey (Fig. 1). At a light intensity of 0.1 lux, the threshold for larvae (23) before rods develop in the retina, the fish deficient in 22:6n-3 still fed less well, but the difference between dietary treatments was similar to that between replicate tanks and was no longer significant. There was little difference in feeding performance between fish reared on the two diets at 1 lux.

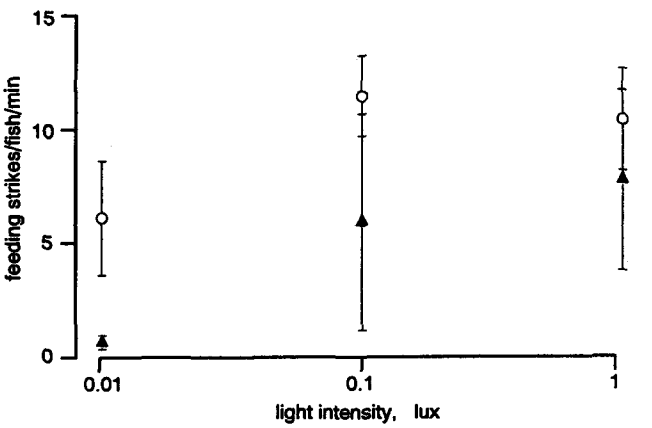


FIG. 1. The effect of light intensity on feeding behavior of herring larvae reared on diets either supplemented with 22:6n-3 (O) or lacking 22:6n-3 (▲). *Symbols represent means ± 95% confidence intervals (n = 6) for* three replicate behavior experiments for each of two rearing groups on each dietary treatment.

Fatty acid and molecular species analysis of the lipids from the eyes of fish from the replicate tanks showed only small differences in composition, none of which were significant ($P > 0.05$). Therefore, in the following presentation, the data for replicate tanks has been combined. Fish reared on the diet lacking 22:6n-3 showed a large decrease in the 22:6n-3 content of total lipid from eyes with concomitant increases in

TABLE 2
The Fatty Acid and di22:6n-3 Phospholipid Content of Eyes from Herring Reared on 22:6n-3-Supplemented and -Deficient Diets (mole %)[a]

	22:6n-3-Supplemented	22:6n-3-Deficient
16:0	19.3 ± 1.6	19.5 ± 0.8
16:1	3.9 ± 0.6	3.9 ± 0.6
18:0	8.4 ± 0.8	8.8 ± 0.4
18:1	15.4 ± 1.0	18.5 ± 0.7
18:2n-6	1.9 ± 0.3	2.7 ± 0.3
20:4n-6	3.8 ± 0.3	5.1 ± 0.3
20:5n-3	9.5 ± 1.0	14.6 ± 1.4
22:5n-3	3.5 ± 0.4	6.6 ± 0.3
22:6n-3	21.0 ± 3.1	6.6 ± 2.6
di22:6n-3	12.0 ± 3.3	2.0 ± 1.1

[a]Mean ± 1 SD, $P < 0.0001$ in all cases. Ten fish were analyzed for fatty acids and 20 for molecular species; 16:1 and 18:1 include both n-9 and n-7 isomers. Only fatty acids present at >2% are shown. 14:0, 15:0, 16:3n-3, 17:0, 18:3n-6, 18:3n-3, 20:1n-9 and n-7, and 20:4n-3 were also present in small amounts.

TABLE 3
The Molecular Species Composition of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), and Phosphatidylserine (PS) from the Eyes of Juvenile Herring Reared on 22:6n-3-Supplemented (control) and 22:6n-3-Deficient (experimental) Diets (mole %)[a]

Molecular species	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine	
	Control	Experimental	Control	Experimental	Control	Experimental
20:5/20:5	trace	0.2 ± 0.1	0.8 ± 0.2	2.9 ± 0.4	trace	0.7 ± 0.2
20:5/22:6	0.4 ± 0.1	trace[b]	3.4 ± 0.5	3.1 ± 0.3	2.6 ± 0.4	2.8 ± 0.3
20:5/22:5	0	0.2 ± 0.1[c]	0	4.0 ± 0.6[a]	0	4.2 ± 0.8[a]
22:6/22:6	5.0 ± 0.7	0.3 ± 0.2[a]	20.8 ± 4.1	2.7 ± 0.6[a]	28.2 ± 5.4	7.6 ± 0.7[b]
22:6/22:5	0.5 ± 0.1	0.2 ± 0.1[c]	5.5 ± 0.7	6.9 ± 0.3	7.6 ± 1.3	8.5 ± 1.4
22:5/22:5	0	1.4 ± 0.2[a]	0	4.4 ± 0.5[a]	0	4.9 ± 0.9[a]
Total diPUFA	5.9 ± 0.8	2.4 ± 0.6	30.5 ± 5.4	26.0 ± 1.6	38.4 ± 6.4	28.7 ± 3.7
16:0/20:5	10.4 ± 1.8	15.7 ± 0.6[c]	6.1 ± 0.8	8.7 ± 0.6[b]	2.9 ± 0.8	5.7 ± 1.2[c]
16:0/22:6	15.0 ± 1.4	3.9 ± 0.4[a]	10.7 ± 1.6	3.2 ± 0.2[a]	5.5 ± 1.2	1.8 ± 0.2[b]
16:0/20:4	4.0 ± 0.3	5.9 ± 0.5[b]	1.7 ± 0.1	2.5 ± 0.2[b]	0.8 ± 0.3	1.7 ± 0.4
16:0/22:5	6.8 ± 1.5	9.1 ± 0.2[a]	5.3 ± 1.0	5.5 ± 0.3	4.9 ± 1.2	5.4 ± 0.7
18:0/20:5	3.5 ± 0.5	6.6 ± 0.3[a]	4.6 ± 0.8	9.5 ± 0.2[a]	2.7 ± 0.7	6.5 ± 0.8[b]
18:0/22:6	10.8 ± 0.9	4.0 ± 0.4[a]	9.6 ± 1.0	4.3 ± 0.4[a]	17.1±1.4	7.2 ± 0.8[a]
18:0/20:4	1.1 ± 0.1	2.0 ± 0.1[a]	2.2 ± 0.3	3.1 ± 0.4[c]	1.6 ± 0.3	4.0 ± 1.9
18:0/22:5	3.9 ± 0.2	4.4 ± 0.1[b]	3.0 ± 0.3	5.1 ± 0.2[a]	7.5 ± 1.4	16.1 ± 0.5[a]
Total SFA/PUFA	51.8 ± 1.6	51.6 ± 0.8	43.2 ± 5.2	41.7 ± 1.3	43.0 ± 6.5	48.8 ± 3.8
16:1/22:6	0.8 ± 0.1	0[a]	1.8 ± 0.4	0[a]	1.1 ± 0.2	0[a]
18:1/20:5	3.2 ± 0.5	5.7 ± 0.3[a]	4.3 ± 0.4	9.8 ± 0.3[a]	1.0 ± 0.0	2.4 ± 0.4[b]
18:1/22:6	1.4 ± 1.1	1.3 ± 0.1	5.9 ± 1.4	3.4 ± 0.4	5.9 ± 1.2	4.3 ± 0.9
18:1/20:4	0	trace	2.2 ± 0.4	3.1 ± 0.4	0.8 ± 0.6	1.1 ± 0.3
18:1/22:5	1.8 ± 1.5	2.3 ± 0.2	3.2 ± 0.5	6.6 ± 0.3[a]	3.8 ± 0.1	7.0 ± 0.5[a]
Total MUFA/PUFA	7.1 ± 1.2	9.3 ± 0.2	17.4 ± 1.7	22.9 ± 0.4	12.6 ± 0.6	14.8 ± 1.5
16:0/16:0	2.8 ± 0.1	2.3 ± 0.2[b]	0.4 ± 0.2	0.2 ± 0.1	0.7 ± 0.2	1.0 ± 0.4
16:0/18:1	19.7 ± 1.4	22.9 ± 0.8[c]	2.5 ± 0.3	2.6 ± 0.6	2.0 ± 0.7	2.6 ± 0.7
18:0/18:1	1.6 ± 0.1	2.0 ± 0.1[c]	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.4	0.8 ± 0.5
18:0/20:1	0.2 ± 0.0	0.2 ± 0.1	0	0	0	0
18:1/18:1	2.3 ± 0.3	2.7 ± 0.2	2.0 ± 0.2	2.5 ± 0.4	1.0 ± 0.3	1.2 ± 0.3
18:1/24:1	0.3 ± 0.1	0.4 ± 0.1	0	0	0	0
Total SFA + MUFA	27.0 ± 1.7	30.5 ± 1.0	5.2 ± 0.6	6.0 ± 0.6	4.4 ± 1.4	5.7 ± 1.9

[a]Significantly different from the 22:6n-3-supplemented fish at [a]$P < 0.0001$, [b]$P < 0.001$, [c]$P < 0.01$. Data are means ± 1 SD of eyes from four fish (two from each replicate tank) matched for size; trace = <0.1%. The order of fatty acids does not imply any stereospecificity on the glycerol. The n-9 and n-7 isomers of monounsaturated fatty acid (MUFA) were not resolved. 20:4 Fatty acid was the n-6 isomer and 22:5 the n-3 isomer. Molecular species containing 18:2n-6 could not be resolved from those containing 22:5n-3. However, 22:5n-3 was two to three times more abundant than 18:2n-6 in both dietary groups (4.0 ± 0.4% vs. 1.8 ± 0.3% and 7.6 ± 0.4% vs. 2.6 ± 0.3% in the 22:6n-3-supplemented and -deficient fish, respectively). PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

20:5n-3 and 22:5n-3 ($P < 0.0001$, all cases) (Table 2). The total glycerophospholipids from eyes of fish on the diet lacking 22:6n-3 had only 17.6% of the di22:6n-3 content of control fish ($P < 0.0001$) (Table 2). The amounts of 18:1, 18:2n-6 and 20:4n-6 were slightly but significantly ($P < 0.0001$) elevated in the eyes of the 22:6n-3-deficient fish (Table 2).

The changes in the n-3 PUFA composition of the lipid from eyes were reflected in large changes in the molecular species of PC, PE, and PS (Table 3). In the diPUFA class of molecular species, di22:6 PC fell from 5.0 to 0.3%, di22:6 PE from 20.8 to 2.7%, and di22:6 PS from 28.2 to 7.6% (Table 3). There was little change in the amounts of 20:5/22:6 and 22:5/22:6 in all three lipid classes, while increases in 20:5/20:5, 20:5/22:5, and 22:5/22:5, particularly in PE and PS, redressed part of the balance. However, the total diPUFA species were decreased in all three lipids classes (Table 3). These changes are summarized in Figure 2.

The other PUFA-containing molecular species, i.e., saturated fatty acid (SFA)/PUFA and monounsaturated fatty acid (MUFA)/PUFA species, followed the same patterns as above with 16:0/20:5, 18:1/20:5, and 18:0/20:5 being elevated in each phospholipid class from the 22:6n-3-deficient fish and 16:0/22:6, 18:1/22:6, and 18:0/22:6 being decreased (Table 3). Although all molecular species containing 22:5n-3 were also elevated, the differences were less marked, and only the changes in 16:0/22:5 and 18:0/22:5 PC and in 18:1/22:5 and 18:0/22:5 PE and PS were significant (Table 3). All arachidonyl-containing molecular species were also elevated in all three phospholipid classes from the 22:6n-3-deficient fish (Table 3), but most changes were small and not significant. In contrast to the marked changes in PUFA-containing molecular species, there was virtually no change in those molecular species containing only SFA and MUFA (Table 3).

The selectivity of particular lipid classes for particular molecular species followed well-established trends found in other studies (6,7,29). Thus, in PC, 16:0/PUFA species were

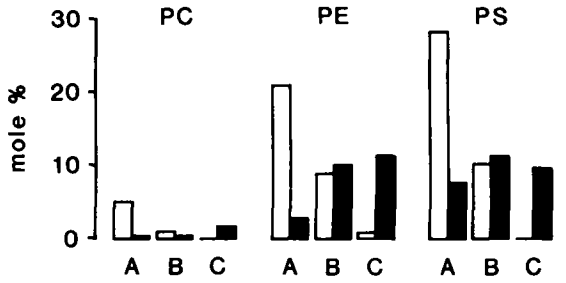


FIG. 2. The dipolyunsaturated fatty acid molecular species composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) from the eyes of juvenile herring fed on diets supplemented with (open bar) or deficient in (filled bars) 22:6n-3. A = 22:6/22:6, B = 20:5/22:6 + 22:5/22:6, C = 20:5/20:5 + 20:5/22:5 + 22:5/22:5.

abundant with low amounts of diPUFA species and a large amount of 16:0/18:1 (Table 3). In PE, 16:0/PUFA species were less prominent, while 18:1/PUFA species were 10–14% more abundant than in PC and diPUFA species contributed 26.0–30.5% (Table 3). PS contained the largest percentage of diPUFA species (28.7–38.4%) in both dietary groups, and in this phospholipid the decrease of 18:0/22:6 was balanced almost completely by an increase in 18:0/22:5 in the fish fed the 22:6n-3-deficient diet.

DISCUSSION

Adult herring have good scotopic vision and feed visually at any time whenever both suitable prey and adequate illumination are available. The present study emphasizes the essential role of 22:6n-3 in the visual process by showing that it is possible to induce a dietary deficiency of 22:6n-3 in predatory, visual feeding fish, so that their ability to feed at low (natural) light intensities is impaired. Though not tested here, predator evasion behavior could also be affected. Late larval and juvenile stages of herring are susceptible to predation by other fish and develop the ability to respond to visual looming stimuli typical of such attacks at the same time as rods appear in the retina (31). A reduction in the ability to detect attacks by predators would markedly decrease the likelihood of survival. The size of the eyes precluded routine preparation of retinas. In adult herring, about 70% of the total phospholipid from eyes was found in retina, and about half the remainder was in the iris muscle which contained half the amount of 22:6n-3 of retina (Bell, M.V., and J.R. Dick, unpublished observations). Retinal phospholipids are therefore the major pool of 22:6n-3 in whole eyes, and even assuming total depletion of 22:6n-3 in all other ocular tissues, the deficit of 22:6n-3 induced in retina in this study would have been substantial.

Herring (22,23) and most other species of marine fish (24) are unable to detect ultraviolet or infrared light; their spectral sensitivity is similar to humans. Herring are totally dependent on photopic vision at first feeding and can only use vision to attack prey at light intensities above 0.1 lux (23). Rods do not occur in significant numbers in herring retinae until eight weeks after hatching when larvae are about 2.5 cm in length and the retinomotor responses of adult fish only develop after this time (19). Adult herring are completely light adapted at 1 lux but are dark adapted, and therefore dependent on scotopic vision, at 0.01 lux (19), the threshold for visual feeding by adults (30). Since rods are recruited to the photoreceptor population from about eight weeks' post-hatching whereas cones are present at hatching, a deficiency of 22:6n-3 during development would be expected to produce a larger effect on scotopic vision than on cone vision. The result found here is consistent with that interpretation. It was not possible to determine in this study whether the decreased proportion of 22:6n-3 in ocular phospholipids was due to decreased amounts in rod outer segment membranes, to a reduced length of the outer segment leading to smaller amounts of outer segment membrane, or to a reduced number of rod cells. The latter two possibilities would also be consistent with a lower sensory threshold. The fact that a substantial part of the deficit of di22:6n-3 molecular species was compensated by increases in molecular species containing 20:5n-3 and 22:5n-3, especially in PE and PS, suggests that the amounts of outer segment membrane were similar in the eyes of both groups of fish. Preliminary microscopic examination of retinas showed rod cells and retinomotor responses present in fish fed on both dietary regimes (Batty, R., and R. Harvey, personal observations). A much more detailed study of retinas in a further experiment would be necessary to eliminate the possibility that a deficiency of 22:6n-3 has an effect on rod development *per se*.

Docosahexaenoic acid is present in a variety of other cell membranes in fish and especially in excitable membranes, including synapses. It is probable therefore that 22:6n-3 was depleted in all other tissues with possible deleterious effects on their functions. This experiment measured the ability of fish to perform a task (feed) at three different light intensities, the lower two chosen to select for vision using cones or rods. Subperformance in consummatory behavior can be due either to a reduced ability to perform the task or to changes in motivational status (or an interaction of the two). A change in motivational status under the more demanding sensory conditions of low light intensity would necessitate light, affecting another neural function directly. Herring larvae become more active in the presence of prey at light intensities below the feeding threshold even though they cannot feed. They are motivated by chemical and mechanical stimulation, but light (vision) is necessary for successful encounter and capture (32). We find it difficult to accept that fish with functioning vision at all light intensities, deprived of food and therefore hungry, might be poorly motivated to feed at low, but natural, light intensities. Difficulties in interpretation are inherent in studies of this nature where the primary events of vision in the retina are closely coupled with signal transduction and processing in the brain where other factors may then modulate responses. Since the 22:6n-3-deficient fish were able to see at higher light intensities, their cone cells and the downstream signal processing from those cells were functional. We believe the

probable lesion in this study lay in the retina since light intensities were specifically chosen which represented the visual threshold for larvae (cones only) and adults (rods and cones). The effect was specific for the lowest light intensity, suggesting impaired scotopic vision either by interrupting the primary events of photon capture by the photoreceptor membranes in rod cells or by the subsequent neural processing of the signal from the rod cells.

Herring, in common with marine fish in general, are unable to synthesise 22:6n-3 from shorter chain C_{18} or C_{20} n-3 PUFA at rates sufficient to meet their requirements. The 22:6n-3-deficient diet also lacked 22:5n-3 and contained only 2.9% 20:5n-3 compared to 11.6% 20:5n-3 in the 22:6n-3-supplemented diet. Consequently, the amounts of 22:5n-3 in the phospholipids from the eyes of the fish reared on the 22:6n-3-deficient diet are notable in that they indicate that herring are able to elongate 20:5n-3 to 22:5n-3, but appear unable to complete the final desaturation step to form 22:6n-3.

The present study has implications for the husbandry of farmed fish and possibly also for natural fish populations. Docosahexaenoic acid is largely absent from marine diatoms; dinoflagellates and prymnesiophytes represent the only substantive source of this fatty acid in marine phytoplankton (33). It is unclear how the species composition and timing of phytoplankton blooms may vary from year to year to influence the amount of 22:6n-3 available (directly and indirectly) to larval marine fish, but amounts of n-3 PUFA in phytoplankton do vary spatially and temporally (34–36). The dietary deprivation of 22:6n-3 in this study was total, and it is very unlikely that this would occur in nature. However, it is unknown whether suboptimal lipid nutrition during early development could ultimately affect recruitment to adult stocks. In herring, rod cells are recruited to the photoreceptor population relatively late in development (19). More recent investigations of other species have shown that rods begin to appear much earlier in development at the first-feeding stage (37) but are very few in number. An adequate dietary supply of 22:6n-3 is probably essential for the survival of marine fish larvae at all stages of development. In mariculture, the supply of adequate amounts of 22:6n-3 to first-feeding larvae is a continuing problem.

Since the fish on the 22:6n-3-deficient diet had impaired vision, the diPUFA molecular species containing 20:5n-3 and 22:5n-3 were unable to replace di22:6n-3 phospholipids functionally in the visual process. At present, the molecular basis for the essentiality of 22:6n-3 in biological processes is not understood. Recent molecular modeling studies have suggested that 22:6n-3-containing glycerophospholipids do not perturb bilayer packing (38,39) as had previously been thought. Furthermore, the favored angle-iron conformation adopted by 22:6n-3 shows the smallest coefficient of thermal expansion of any fatty acid (40), making these lipids particularly suited to control bilayer volume. Finally, it has been proposed that 22:6n-3-containing glycerophospholipids, and especially di22:6n-3 molecular species, provide a highly structured liquid–crystalline membrane which can accommodate the rapid conformational changes in rhodopsin associated with the light-transducing process (41,42). In recombinant bovine rod outer segment membranes, an equimolar mixture of PE and PC containing 50 mole % docosahexaenoyl chains resulted in optimal photochemical function of rhodopsin (43). It was interesting in the present study to find that PS retained 22:6n-3- containing molecular species, and especially di22:6n-3, better than PE even though the % of PS (8.1%) in total phospholipid of the eye is much less than that of PE (26.5%) (44). The total diPUFA molecular species was highest in PS from both dietary groups and the amounts of 20:5/22:5, 22:5/22:6, and 22:5/22:5, as well as 22:6/22:6, were higher in PS than in PE from the eyes of the 22:6n-3-deficient fish. Few studies have been carried out on the physical–chemical properties of PS since it is a minor component of most membranes. However, Hubbell (45) suggested that the asymmetric distribution of PS in the disc membrane was caused by interaction with rhodopsin.

Irrespective of its precise mode of action, 22:6n-3 appears to have a unique role in vertebrate rod function. Clearly, any explanation of the particular role of 22:6n-3 in the visual process must encompass the unusual prominence of di22:6n-3 molecular species of both PS and PE. Experiments, such as the present one, establish that marine fish larvae offer particular advantages for dietary intervention studies on the role of this fatty acid in vertebrate vision.

ACKNOWLEDGMENT

We thank R. Harvey for carrying out microscopic examination of retinas.

REFERENCES

1. Fliesler, S.J., and Anderson, R.E. (1983) "Chemistry and Metabolism of Lipids in the Vertebrate Retina," *Prog. Lipid Res. 22*, 79–131.
2. Sastry, P.S. (1985) "Lipids of Nervous Tissue: Composition and Metabolism," *Prog. Lipid Res. 24*, 69–176.
3. Poulos, A., Brown-Woodman, P.D.C., White, I.G., and Cox, R.I. (1975) "Changes in Phospholipids of Ram Spermatozoa During Migration Through the Epididymis and Possible Origin of Prostaglandin F2α in Testicular and Epididymal Fluid," *Biochem. Biophys. Acta 388*, 12–18.
4. Louie, K., Wiegand, R.D., and Anderson, R.E. (1988) "Docosahexaenoate-Containing Molecular Species of Glycerophospholipids from Frog Retinal Rod Outer Segments Show Different Rates of Biosynthesis and Turnover," *Biochemistry 27*, 9014–9020.
5. Stinson, A.M., Wiegand, R.D., and Anderson, R.E. (1991) "Recycling of Docosahexaenoic Acid in Rat Retinas During n-3 Fatty Acid Deficiency," *Exp. Eye Res. 52*, 213–218.
6. Bell, M.V., and Dick, J.R. (1991) "Molecular Species Composition of the Major Diacyl Glycerophospholipids from Muscle, Liver, Retina and Brain of Cod (*Gadus morhua*)," *Lipids 26*, 565–573.
7. Bell, M.V., and Dick, J.R. (1993) "The Appearance of Rods in the Eyes of Herring and Increased di-Docosahexaenoyl Molecular Species of Phospholipids," *J. Mar. Biol. Ass. U.K. 73*, 679–688.
8. Sargent, J.R., Henderson, R.J., and Tocher, D.R. (1989) "The Lipids," in *Fish Nutrition* (Halver, J.E., ed.) 2nd edn., pp.153–218, Academic Press, San Diego.

9. Rivers, J.P.W., Sinclair, A.J., and Crawford, M.A. (1975) "Inability of the Cat to Desaturate Essential Fatty Acids," *Nature 285*, 171 -173.
10. Neuringer, M., Anderson, G.J., and Connor, W.E. (1988) "The Essentiality of n-3 Fatty Acids for the Development and Function of the Retina and Brain," *Ann. Rev. Nutr. 8*, 517–541.
11. Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H. (1991) "The Metabolism of 7,10,13,16,19-Docosapentaenoic Acid to 4,7,10,13,16,19-Docosahexaenoic Acid in Rat Liver is Independent of a 4-Desaturase," *J. Biol. Chem. 266*, 19995–20000.
12. Neuringer, M., Connor, W.E., Van Petten, C., and Barstad, L. (1984) "Dietary Omega-3 Fatty Acid Deficiency and Visual Loss in Infant Rhesus Monkeys," *J. Clin. Invest. 73*, 272–276.
13. Enslen, M., Milon, L., and Malnoe, A. (1991) "Effect of Low Intake of n-3 Fatty Acids During Development on Brain Phospholipid Fatty Acid Composition and Exploratory Behavior in Rats," *Lipids 26*, 203–208.
14. Uauy, R.D., Birch, D.G., Birch, E.E., Tyson, J.E., and Hoffman, D. (1990) "Effect of Dietary Omega-3 Fatty Acids on Retinal Function of Very-Low-Birth-Weight Neonates," *Ped. Res. 28*, 485–492.
15. Uauy, R., Birch, E., Birch, D., and Peirano, P. (1992) "Visual and Brain Function Measurements in Studies of n-3 Fatty Acid Requirements of Infants," *J. Pediat. 120*, S168–S180.
16. Innis, S.M. (1991) "Essential Fatty Acids in Growth and Development," *Prog. Lipid Res. 30*, 39–103.
17. Anon. (1992) *Unsaturated Fatty Acids. Nutritional and Physiological Significance*, British Nutrition Foundation report, pp.156–157, Chapman Hall, London.
18. Wainwright, P.E. (1992) "Do Essential Fatty Acids Play a Role in Brain and Behavioural Development?" *Neurosci. Biobehav. Rev. 16*, 193–205.
19. Blaxter, J.H.S., and Jones, M.P. (1967) "The Development of the Retina and Retinomotor Responses in the Herring," *J. Mar. Biol. Ass. U.K. 47*, 677–697.
20. Navarro, J.C., Batty, R.S., Bell, M.V., and Sargent, J.R. (1993) "Effects of Two *Artemia* Diets with Different Contents of Polyunsaturated Fatty Acids on the Lipid Composition of Larvae of Atlantic Herring (*Clupea harengus* L.)," *J. Fish Biol. 43*, 503–515.
21. Blaxter, J.H.S., and Batty, R.S. (1985) "Herring Behaviour in the Dark: Responses to Stationary and Continuously Vibrating Obstacles," *J. Mar. Biol. Ass. U.K. 65*, 1031–1049.
22. Blaxter, J.H.S. (1964) "Spectral Sensitivity of the Herring *Clupea harengus* L.," *J. exp. Biol. 41*, 155–162.
23. Blaxter, J.H.S. (1968) "Visual Thresholds and Spectral Sensitivity of Herring Larvae," *J. exp. Biol. 48*, 39–53.
24. Douglas, R.H., and Hawrshyn, C.W. (1990) "Behavioural Studies of Fish Vision: An Analysis of Visual Capabilities," in *The Visual System of Fish*, Douglas (R.H., and Djamgoz, M.B.A., eds.) pp. 373–418, Chapman and Hall, London.
25. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) "A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues," *J. Biol. Chem. 226*, 497–509.
26. Henderson, R.J., Bell, M.V., Park, M.T., Sargent, J.R., and Falcon, J. (1994) "Lipid Composition of the Pineal Organ from Rainbow Trout (*Oncorhynchus mykiss*)," *Lipids 29*, 311-317.
27. Takamura, H., and Kito, M. (1991) "A Highly Sensitive Method for Quantitative Analysis of Phospholipid Molecular Species by High-Performance Liquid Chromatography," *J. Biochem. 109*, 436–439.
28. Vitiello, F., and Zanetta, J.-P. (1978) "Thin-layer Chromatography of Phospholipids," *J. Chromatogr. 166*, 637–640.
29. Bell, M.V., and Tocher, D.R. (1989) "Molecular Species Composition of the Major Phospholipids in Brain and Retina from Rainbow Trout (*Salmo gairdneri*) Occurrence of High Levels of di-(n-3) Polyunsaturated Fatty Acid Species," *Biochem. J. 264*, 909–915.
30. Batty, R.S., Blaxter, J.H.S., and Richard, J.M. (1990) "Light Intensity and the Feeding Behaviour of Herring, *Clupea harengus*," *Mar. Biol. 107*, 383–388.
31. Batty, R.S. (1989) "Escape Response of Herring Larvae to Visual Stimuli," *J. Mar. Biol. Ass. U.K. 69*, 647–654.
32. Batty, R.S. (1987) "Effect of Light Intensity on Activity and Food-Searching of Larval Herring, *Clupea harengus:* A Laboratory Study," *Mar. Biol. 94*, 323-327.
33. Sargent, J.R., Parkes, R.J., Mueller-Harvey, I., and Henderson, R.J. (1987) "Lipid Biomarkers in Marine Ecology," in *Microbes in the Sea* (Sleigh, M.A., ed.) pp. 119–138, Ellis Horwood Ltd., Chichester.
34. Kattner, G., Gercken, G., and Eberlein, K. (1983) "Development of Lipids During a Spring Plankton Bloom in the Northern North Sea," *Mar. Chem. 14*, 149–162.
35. Fraser, A.J., Sargent, J.R., Gamble, J.C., and Seaton, D.D. (1989) "Formation and Transfer of Fatty Acids in an Enclosed Marine Food Chain Comprising Phytoplankton, Zooplankton and Herring (*Clupea harengus* L.) Larvae," *Mar. Chem. 27*, 1–18.
36. Pond, D., Priddle, J., Sargent, J.R., and Watkins, J.L. (1993) in *University Research in Antarctica, 1989–1992*. (Heywood, R.B., ed.) pp. 133–139, British Antarctic Survey, Cambridge.
37. Powers, M.K., and Raymond, P.A. (1990) "Development of the Visual System," in *The Visual System of Fish*. (Douglas, R.H. Douglas, and Djamgoz, M.B.A.) pp. 419–442, Chapman and Hall, London.
38. Applegate, K.R., and Glomset, J.A. (1986) "Computer-Based Modeling of the Conformation and Packing Properties of Docosahexaenoic Acid," *J. Lipid Res. 27*, 658–680.
39. Applegate, K.R., and Glomset, J.A. (1991) "Effect of Acyl Chain Unsaturation on the Packing of Model Diacylglycerols in Simulated Monolayers," *J. Lipid Res. 32*, 1645–1655.
40. Rabinovitch, A.L., and Ripatti, P.O. (1991) "On the Conformational, Physical Properties and Functions of Polyunsaturated Acyl Chains," *Biochim. Biophys. Acta 1085*, 53–62.
41. Salem, N., Kim, H.-Y., and Yergey, J.A. (1986) "Docosahexaenoic Acid: Membrane Function and Metabolism," in *Health Effects of Polyunsaturated Fatty Acids in Seafoods* (Simopoulos, A.P., ed.) pp. 263–317, Academic Press, New York.
42. Sargent, J.R., Bell, M.V., and Tocher, D.R. (1993) "Docosahexaenoic Acid and the Development of Brain and Retina in Marine Fish," in *Omega-3 Fatty Acids: Metabolism and Biological Effects* (Drevon, C.A., Baksaas I., and Krokan, H.E., eds.) pp. 139–149, Birkhauser Verlag Basel/Switzerland.
43. Wiedmann, T.S., Pates, R.D., Beach, J.M., Salmon, A., and Brown, M.F. (1988) "Lipid–Protein Interactions Mediate the Photochemical Function of Rhodopsin," *Biochemistry 27*, 6469–6474.
44. Tocher, D.R., and Harvie, D.G. (1988) "Fatty Acid Compositions of the Major Phosphoglycerides from Fish Neural Tissues; (n-3) and (n-6) Polyunsaturated Fatty Acids in Rainbow Trout (*Salmo gairdneri*) and Cod (*Gadus morhua*) Brains and Retinas," *Fish Physiol. Biochem. 5*, 229–239.
45. Hubbell, W.L. (1990) "Transbilayer Coupling Mechanism for the Formation of Lipid Asymmetry in Biological Membranes. Application to the Photoreceptor Disc Membrane," *Biophys. J. 57*, 99–108.

[Received November 21, 1994, and in revised form February 24, 1995; Revision accepted March 6, 1995]

Eicosanoid Generating Capacities of Different Tissues from the Rainbow Trout, *Oncorhynchus mykiss*

John Knight, Jason W. Holland, Linda A. Bowden, Katrina Halliday and Andrew F. Rowley*

School of Biological Sciences, University of Wales, Swansea, Singleton Park, Swansea, SA2 8PP, United Kingdom

ABSTRACT: The eicosanoid generating potential of the brain, gills, skin, ovary, muscle, eye, liver, spleen, heart, and alimentary canal in the rainbow trout, *Oncorhynchus mykiss*, was examined. All the organs/tissues examined synthesized the 12-lipoxygenase products, 12-hydroxyeicosatetraenoic acid (12-HETE), and 12-hydroxyeicosapentaenoic acid (12-HEPE), implying the widespread nature of this enzyme in trout. Both prostaglandin E and LTC were also found in variable amounts in the organs, with the greatest amount of PGE found in the gill. Leukotriene (LT) B_4 and LTB_5 were found in supernatants from calcium ionophore-challenged brain, skin, ovary, liver, spleen, and heart, but the lipoxins A_4 and A_5 were only present in brain, ovary, and spleen in relatively small amounts. As lipoxins have previously been shown to be synthesized by macrophages in rainbow trout [Pettitt *et al., J. Biol. Chem. 266,* 8720–8726 (1991)], and related cells (microglial cells) are found in the brain of mammals, the localization of macrophage-like cells in trout brain was investigated immunocytochemically. Monoclonal antibodies specific for trout leucocytes failed to identify any microglial-like cells in sections of the brain, although microvessels containing immuno-positive reaction products were observed. A number of distinct lipoxygenase products were found in supernatants of ionophore-challenged gill, including 14-hydroxydocosahexaenoic acid, 12-HETE, and 12-HEPE, and a large number of dihydroxy fatty acid derivatives with conjugated triene chromophores. One of these products was tentatively identified as 8(*R*),15(*S*)-dihydroxyeicosatetraenoic acid, a dual 12- and 15-lipoxygenase product, but apparently no LTB_4 was generated by this tissue.

Lipids 30, 451–458 (1995).

The biosynthesis and role of eicosanoids, such as leukotrienes (LT), lipoxins (LX), and prostaglandins (PG) have been extensively studied in mammals. In particular, LTB_4 and PGE_2 have been shown to play important roles in inflammation and immune regulation (1,2), whereas PGs, in general, are also involved in homeostatic and reproductive processes (3).

Despite this wealth of information on eicosanoids in mammals, significantly less is known of the generating capacity of tissues in other animals, including fish. Fish tissues, unlike their mammalian counterparts, often contain relatively large amounts of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) as well as the arachidonic acid (AA; 20:4n-6) characteristic of mammalian tissues (4). As all of these fatty acids can act as substrates for eicosanoid generation (5), fish have been reported to generate a plethora of lipoxygenase products including LXA_4 and LTB_4 and the equivalent 5-series compounds derived from EPA (6,7) and cyclooxygenase products of the 2- and 3-series derived from AA and EPA, respectively (8). The tissue most commonly studied in fish is blood (6–10), although the eicosanoid-generating capacity of the gills (5,11–14), skin (15), gonads (16–18), heart (19), and kidney (20) have also been examined. To date, however, no study has attempted to define which eicosanoids are synthesized by an extensive range of tissues/organs in a single species of fish.

Therefore, this study was undertaken to compare the eicosanoid-generating ability of a wide range of tissues/organs (gill, heart, spleen, alimentary canal, eye, skin, muscle, liver, ovary, and brain) in the rainbow trout, *Oncorhynchus mykiss.*

MATERIALS AND METHODS

Fish. Rainbow trout (*O. mykiss*), typically between 120–500 g, were used in this study and purchased from local fish farms. They were maintained as described previously (6,7).

Preparation of tissues for calcium ionophore challenge. Fish were killed by overdose in ethyl aminobenzoate (0.05 $g.L^{-1}$) and exsanguinated *via* the caudal vessel. The main body cavity was exposed, and the alimentary canal removed intact and placed in a large beaker containing ice-cold Ca/Mg-free Hank's balanced salt solution (HBSS). The liver

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; EIA; enzyme immunoassay; EPA; eicosapentaenoic acid; DHA, docosahexaenoic acid; FLAP, 5-lipoxygenase activating protein; HBSS, Hank's balanced salt solution; HDHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; LX, lipoxin; NDGA, nordihydroguiaretic acid; PBS, phosphate-buffered saline; PG(s), prostaglandin(s); RP-HPLC, reverse-phase high-performance liquid chromatography.

Copyright © 1995 by AOCS Press

and any associated fat was excised from the alimentary canal and a number of pyloria caeca (*ca.* 5–8) were removed from the intestine and cut into small (0.5-mm long) pieces. The esophagus, stomach, and small and large intestine were isolated, and small pieces (*ca.* 1-cm long) were washed thoroughly in Ca/Mg-free HBSS to remove all gut contents. Portions of ovary and spleen were also excised and placed in Ca/Mg-free HBSS.

The thoracic region of the fish was exposed, and *ca.* 500 μL of ice-cold Ca/Mg-free HBSS was injected into the ventricle of the heart. This procedure invariably restarted the heart beating, flushing out any residual blood from the other chambers of the heart and gills. The heart was then excised, and a gill arch removed and gill filaments cut into small fragments and washed free of contaminating blood by centrifugation (1000 × *g*, 10 min at 4°C). The eyes were excised using a small pair of scissors and connective tissue and surrounding muscle removed, while the brain was removed from the cranium intact and placed in cold Ca/Mg-free HBSS. Finally, an area of integument was washed with distilled water to remove any mucus, and a small rectangular area was scored out with a scalpel. This was subsequently peeled away from the underlying musculature and cleaned to remove fragments of muscle. A small amount of muscle from below this area was also excised using a sterile scalpel.

All organs/tissues were weighed, and soft tissues (brain, spleen, and ovary) teased through a fine mesh into 5 mL of ice-cold Ca/Mg-free HBSS to obtain cell suspensions. The remaining, more consolidated tissues were cut into small pieces (1–2 mm^3) in Ca/Mg-free HBSS. All samples were transferred into plastic centrifuge tubes and centrifuged at 2800 × *g* for 5 min at 4°C, and the pellets resuspended in 5 mL of Ca/Mg-containing (0.26 $g.L^{-1}CaCl_2 \cdot 2H_2O$, 0.41 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$) HBSS, and the samples challenged with 5 μM calcium ionophore, A23187 (Sigma Chemical Co. Ltd., Poole, United Kingdom) for 20 min at 18°C. Supernatants were obtained following centrifugation (2800 × *g*, 5 min, 4°C) to remove cell debris, and were either stored at −70°C or extracted immediately for reverse-phase high-performance liquid chromatographic (RP-HPLC) analysis.

Eicosanoid analysis. PGE and LTC were quantified by enzyme immunoassay (EIA), and all other lipoxygenase products were separated by RP-HPLC. For EIA, supernatants from ionophore-challenged tissues were tested with commercially-available EIA kits (Cascade Biochem Ltd., Reading, United Kingdom; and Cayman Chemicals, Ann Arbor, MI) as directed in the protocols supplied. For PGE_2, the sensitivity of the assay was 20 $pg.mL^{-1}$ with 100% cross-reactivity with PGE_3, and for LTC_4 the sensitivity was 10 $pg.mL^{-1}$ with 100% cross-reactivity with LTC_5 and <0.01% cross-reactivity with $LTB_{4/5}$.

Samples for RP-HPLC analysis were Sep-Pak extracted, as described previously (6,7), after the addition of 100 ng of PGB_2 (Sigma Chemical Co. Ltd.) as an internal standard. Separation was achieved using an Ultrasphere C_{18} ODS column (250 × 4.6 mm; Beckman Instruments, High Wycombe, United Kingdom) and a linear gradient changing from either 100% water/methanol/acetonitrile/acetic acid (45:30:25:0.05, by vol, pH 5.7) to 100% methanol over 40 min with a flow rate of 0.6 mL min^{-1} or water/methanol/acetic acid (35:65:0.05, by vol, pH 5.7) to 100% methanol over 40 min with flow rates of either 0.6 or 0.8 mL min^{-1}. The eluted compounds were detected with either a Beckman 167 dual wavelength detector or a Waters 991 diode array detector (Waters Chromatography, Northwich, Cheshire, United Kingdom). Identification of products generated was by characteristic peak absorption spectra, co-chromatography with authentic standards [LXA_4, 11-*trans*-LXA_4, LXA_5, LTB_4, LTB_5, 6-*trans*-12-*epi*-LTB_4, 6-*trans*-LTB_4, 8(*R*),15(*S*)DiHETE, 5(*S*), 6(*R*)DiHETE, 5(*S*),12(*S*)DiHETE, 5-HETE, 12-HETE, and 12-HEPE (HETE = hydroxyeicosatetraenoic acid; HEPE = hydroxyeicosapentaenoic acid) Cascade Biochem Ltd., Reading, United Kingdom] or by reference to previous gas chromatography/mass spectrometry studies (6,7). Quantification of the principal products formed was by reference to the internal standard (PGB_2) using published extinction coefficients.

Effect of captopril, nordihydroguiaretic acid (NDGA), esculetin, MK886, and exogenous fatty acids on lipoxygenase product generation by trout gill. To investigate the mechanism of eicosanoid generation in trout gill further, either homogenates or chopped gill tissue were incubated in the presence of the lipoxygenase inhibitors, esculetin, and NDGA (1–100 μM), or the LTA hydrolase inhibitor, captopril (10–200 μM), or the 5-lipoxygenase activating protein (FLAP) inhibitor, MK886 (10–1000 ng mL^{-1}). Additionally, gill homogenates were incubated with exogenous AA, EPA, or DHA (25–150 μM) for 10 min at 18°C. Gill homogenates were prepared by removal of gill arches from the filaments and subsequent homogenization of *ca.* 0.3 g of filament in 4 mL of ice-cold Ca/Mg-containing HBSS. The supernatants were used immediately following centrifugation (10,000 × *g* for 5 min). Product generation was assessed by RP-HPLC.

Immunocytochemical analysis of trout brain. As previous studies have reported the presence of leucocytes in the brain of both mammals (21) and fish (22) and the generation of eicosanoids by these or related cells in mammalian brain (23), the distribution of leucocyte-like cells in the brain of *O. mykiss* was determined using monoclonal antibodies, 21F11 and 21G6, which have been shown to cross-react with all trout leucocytes but not with thrombocytes (platelet equivalents of fish) and erythrocytes (24). Monoclonal antibodies were produced using a standard methodology (25) by fusion of NS1 myeloma cells with splenocytes from mice immunized with formalin-fixed [1% formalin in phosphate-buffered saline (PBS)] purified trout macrophages. Following initial dilution of the hydridomas, these were screened for their specificity by ELISA and immunocytochemistry using monolayers of peripheral blood leucocytes as detailed previously (24). Selected hybridomas were cloned down by limiting dilution, rescreened and selected clones grown in RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 $iu.mL^{-1}$ penicillin, and 0.1 $mg.mL^{-1}$ streptomycin and stored at −70°C prior to use.

Trout brains were excised and washed free of any contaminating blood with Ca/Mg-free HBSS and fixed in ice-cold 4% Baker's formol-calcium, pH 7.0, for 12–24 h. Dehydrated material was embedded in Paraplast histological wax and sections (*ca.* 10-μm thick) cut and attached to glass slides. Sections of brain were rehydrated and incubated in 3% H_2O_2 in PBS for 30 min to inactivate endogenous peroxidase. Following extensive washing (× 3 in PBS), sections were incubated in blocking solution (10% normal goat serum, 3% bovine serum albumin, 0.1 M ammonium chloride, 0.05% Tween-20, 0.1 M glycine in PBS) for 30 min at room temperature (RT). Subsequently, they were incubated in a hybridoma supernatant (21F11 or 21G6) blocking solution mixture (1:1 ratio) for 60 min at RT, washed in PBS (×3), and incubated in goat antimouse Ig antiserum conjugated to peroxidase (1:1000 dilution in blocking solution) for 60 min at RT, washed in PBS (×3), and incubated in the dark in 0.05% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in PBS for 15 min. Finally, sections were rinsed in PBS (×3), and some counterstained with Cole's haematoxylin (4 min) prior to mounting in a water-soluble mountant. As controls, additional sections of brain were incubated in the absence of both primary and secondary antibodies or with primary or secondary antibodies alone followed by the stages described here.

Statistical analyses. All quantitative results are expressed as a mean ± SD or SE and, where appropriate, compared using Student's *t*-test for related samples.

RESULTS

All organs/tissues examined were found to generate 12-HETE, 12-HEPE, PGE, and LTC immunoreactive material (Table 1). The greatest amount of PGE immunoreactive material was found in the brain and this organ also generated LXA_4 and LXA_5 in significant amounts (Fig. 1 and Table 1). As previous studies have reported LX generation in mononuclear phagocytes both in mammals and fish (6,7,26), and microglial cells in mammalian brain are derived from monocytes, the presence of such cells in the brain of rainbow trout was investigated using monoclonal antibodies (21F11 and 21G6). No cells were observed in histological sections of the brain with the characteristic stellate appearance of microglial cells, and the immunoperoxidase-positive staining was limited to capillary-like vessels containing 21F11/21G6$^+$ material (Fig. 2).

Despite the highly vascularized nature of the gills, no LXs were found in the supernatants of ionophore-challenged material (Table 1). However, gills were found to yield the largest amount of LTC-immunoreactive material of all tissues examined, as well as significant levels of monohydroxy fatty acids, 12-HETE and 12-HEPE (Fig. 3 and Table 1). One further product with a long retention time and a λmax of 235 nm, characteristic of conjugated diene-containing fatty acid derivatives, was also observed in the ionophore-challenged gill (Fig. 3). Incubation of gill homogenates with exogenous DHA caused a dose-dependent increase in this product but had no significant effect on 12-HETE and 12-HEPE generation (Fig. 4), suggesting that it is probably 14-hydroxy-4,7,10,13,16,19-docosahexaenoic acid (14-HDHE), previously shown to be synthesized in fish gill by the action of 12-lipoxygenase activity on this substrate (5,12). The generation of this compound and the other monohydroxy fatty acids, 12-HETE and 12-HEPE, was inhibited by the specific lipoxygenase inhibitors, esculetin (IC_{50} 7.5 μM) and NDGA (IC_{50} 5.0 μM). A variable number of di-HETEs/di-HEPEs with conjugated trienes and λmax 268–275 nm were also found in supernatants of ionophore-challenged gill, and some of these were apparently only found in gill (Figs. 3 and 5). The most abundant of these compounds had a retention time of *ca.* 22 min and had a λmax of 275 nm (peak 8 on Fig. 3), but failed to co-chromatograph with any of the authentic standards employed. The second largest peak had a retention time of 19.5 min, a λmax of 268 nm, and co-chromatographed with authentic 8(*R*),15(*S*)di-HETE (Figs. 3 and 5). Furthermore, the online ultraviolet scans of the material under this peak (peak 6) were also similar to authentic 8(*R*),15(*S*)di-HETE in terms

TABLE 1
Eicosanoid Generation by Ionophore-Stimulated Rainbow Trout Tissue as Measured by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) and Enzyme Immunoassay

	Product (ng/mg wet wt)[a]							
Tissue	LXA_5	LXA_4	LTB_5	LTB_4	12-HEPE	12-HETE	PGE	LTC
Brain	0.45 ± 0.01[b]	0.162 ± 0.007	0.042 ± 0.027	0.016 ± 0.003	1.79 ± 0.71	1.08 ± 0.31	0.70 ± 0.12	0.03 ± 0.01
Gills	—	—	*[c]	*[c]	4.07 ± 0.48	4.59 ± 0.82	0.17 ± 0.02	0.17 ± 0.01
Skin	—	—	0.281 ± 0.048	0.093 ± 0.005	4.40 ± 1.69	3.62 ± 1.94	0.08 ± 0.02	0.15 ± 0.04
Ovary	0.002 ± 0.002	0.011 ± 0.008	0.007 ± 0.008	0.009 ± 0.009	0.43 ± 0.09	0.17 ± 0.04	0.02 ± 0.02	0.04 ± 0.01
Muscle	—	—	—	—	0.40 ± 0.09	0.35 ± 0.15	0.03 ± 0.01	0.03 ± 0.04
Eye	—	—	—	—	0.02 ± 0.01	0.51 ± 0.11	0.06 ± 0.04	0.02 ± 0.01
Liver	—	—	0.022 ± 0.007	0.019 ± 0.010	0.29 ± 0.09	0.28 ± 0.09	0.21 ± 0.12	0.09 ± 0.01
Spleen	0.041 ± 0.005	0.188 ± 0.086	0.312 ± 0.101	0.319 ± 0.177	0.52 ± 0.20	0.49 ± 0.21	0.32 ± 0.14	0.10 ± 0.01
Heart	—	—	0.045 ± 0.011	0.037 ± 0.017	0.10 ± 0.03	0.20 ± 0.05	0.45 ± 0.09	0.12 ± 0.05

[a]LX, lipoxin; LT, leukotriene; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin. —, Not detected by RP-HPLC.
[b]Mean values ± SD, n = 3–5.
[c]See Results section.

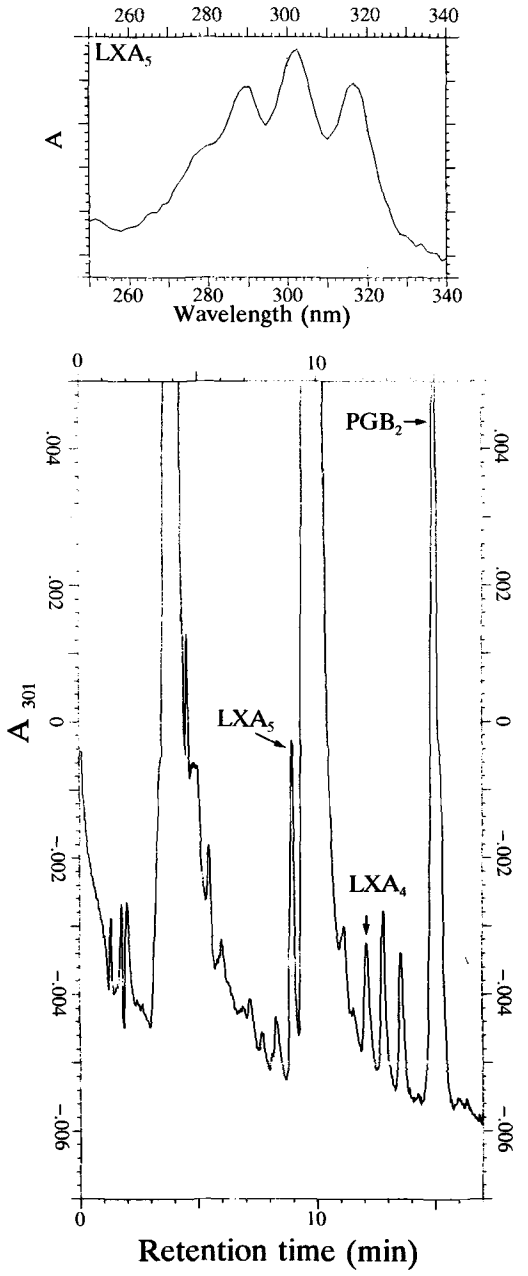


FIG. 1. Reverse-phase high-performance liquid chromatographic chromatogram demonstrating the generation of LXA_4 and LXA_5 by rainbow trout brain challenged with calcium ionophore. Upper inset shows the ultraviolet scan of the material under the peak identified as LXA_5 revealing the characteristic conjugated chromophore with a λmax of 301 nm and shoulders at 289 and 317 nm. The large unlabelled peak with a retention time of *ca.* 10 min is a contaminant from the incubation procedure and is not a product of brain tissue, and the peak with a retention time of *ca.* 4 min represents the solvent front. PG, prostaglandin.

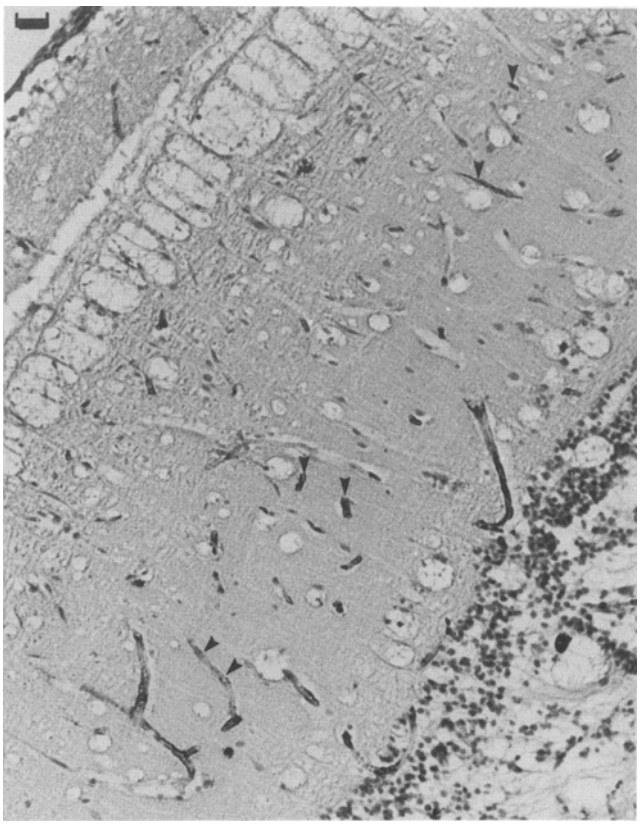


FIG. 2. Section through the optic lobe region of trout brain showing the immunocytochemical localization of $21G6^+$ material in vessels (unlabelled arrows). Scale bar = 25 μm.

of λmax, position of shoulders, and overall slope (Fig. 5). One further main peak had a retention time of 21 min and, although the material under the peak co-chromatographed with LTB_4 in the two gradient solvent systems employed (see Materials and Methods section) its λmax was *ca.* 272 nm, which is different from the ultraviolet profile of authentic LTB_4 that has a λmax of 270 nm. Furthermore, the production of this material was relatively insensitive to captopril (an LTA_4 hydrolase inhibitor, see Ref. 27) at all concentrations tested and the FLAP inhibitor, MK-886. Finally, there were also a number of smaller peaks (e.g., peaks 2 and 5 in Fig. 3) apparently containing di-HETEs/di-HEPEs, but these were not present in all gill samples examined and so were not investigated further.

Of the remaining tissues examined, only ovary and spleen generated LXs, while the skin, ovary, liver, spleen, heart, and alimentary canal produced LTB_4 and LTB_5. The amounts of LTB_4 and LTB_5 synthesized by different regions of the alimentary canal (esophagus, stomach, pyloric caeca, anterior, and posterior intestine) were all similar, whereas in the case of LTC, significantly more product was generated by the stomach than the pyloric caeca and the posterior intestine ($P < 0.05$; Fig. 6). The levels of PGE-immunoreactive material also differed within the regions of the alimentary canal tested with the pyloric caeca, and the anterior and posterior intestine generating statistically significant ($P < 0.005$) more product than the esophagus and stomach. No similar distinct trend was observed for 12-HETE and 12-HEPE, although the anterior intestine was found to synthesize significantly less 12-HETE than the posterior intestine ($P < 0.05$).

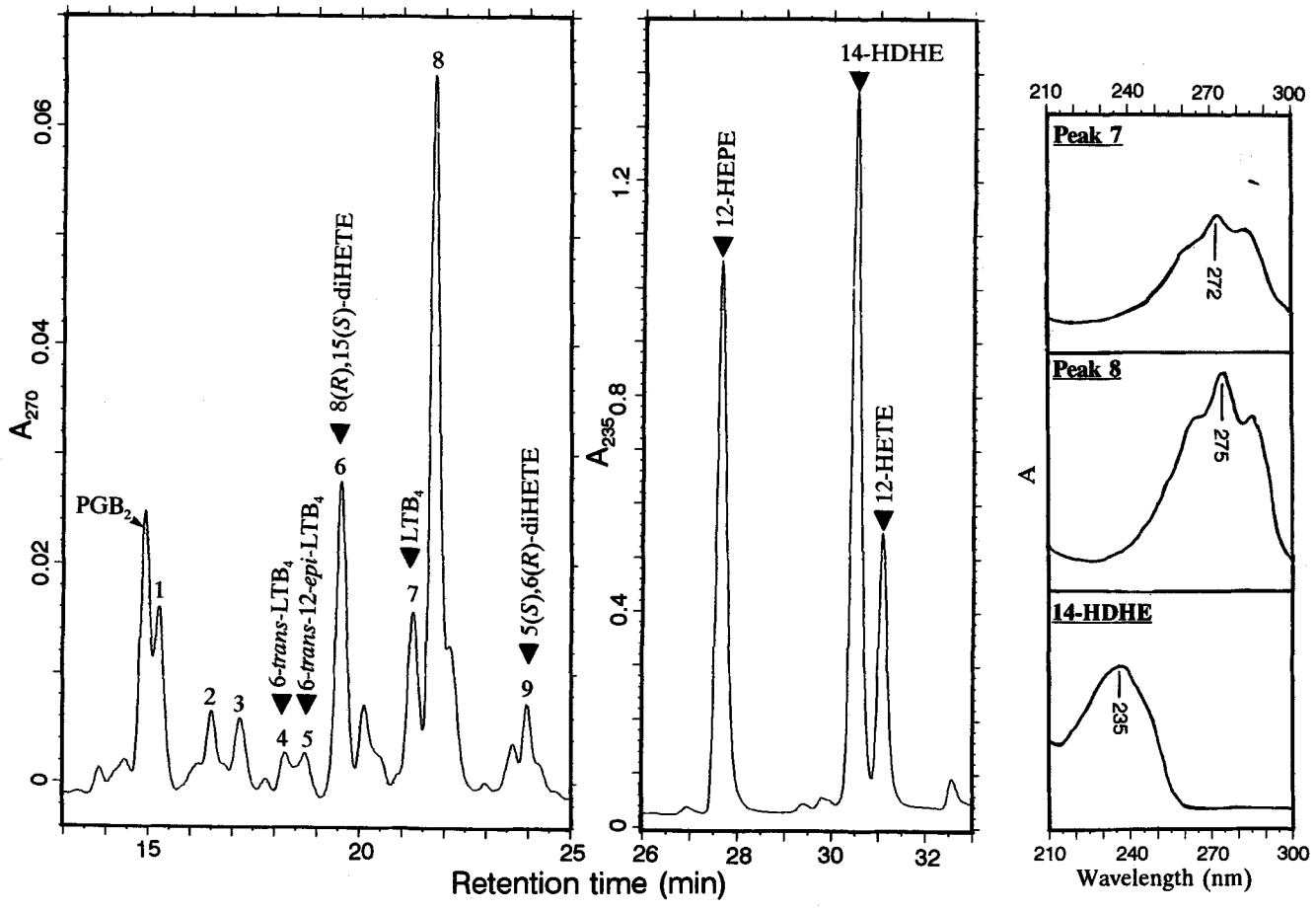


FIG. 3. Representative reverse-phase high-performance liquid chromatographic chromatogram showing the products generated by trout gill after challenge with calcium ionophore, A23187. Peaks labelled 1–9 all contain material with conjugated triene chromophores. The retention times of authentic di-HETEs/LTB_4 (indicated by the position of the arrow heads) are shown for comparison. Insets show ultraviolet scans of material under some of the main peaks. LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HDHE, hydroxydocosahexaenoic acid.

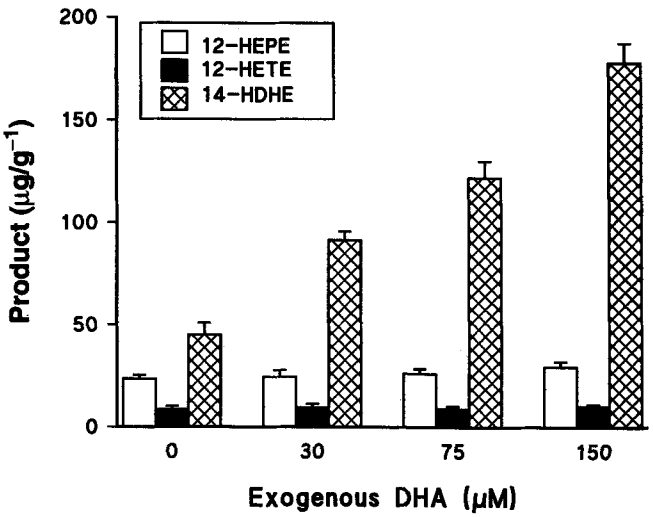


FIG. 4. Effect of exogenous docosahexaenoic acid (DHA) on the generation of 12-HETE, 12-HEPE, and material tentatively identified as 14-HDHE by trout gill homogenate. Homogenates were incubated for 10 min at 18°C in the presence of 0, 30, 75, or 150 μM DHA, and the eicosanoids generated were Sep-Pak extracted, separated, and quantified by reverse-phase high-performance liquid chromatography. Values are means ± SE, n = 3. Abbreviations as in Figure 3.

DISCUSSION

All of the organs/tissues examined in the present study generated the 12-lipoxygenase products, 12-HEPE and 12-HETE, implying that this enzyme is widely distributed in rainbow trout. Previous studies have also reported the preponderance of 12-lipoxygenase products in both the gills (11,12) and in the blood cells (6,7,9,24) of a range of fish, and this enzyme has been partially purified from trout gill (11) and its substrate specificity determined (5). German *et al.* (5) showed, using kinetic analysis of 12-lipoxygenase toward AA and DHA, that this enzyme displays a lack of substrate specificity. Hence, in gill, at least, both 12-HETE and 12-HEPE, as well as the equivalent product from DHA, 14-HDHE, are synthesized. The ability of gill to produce monohydroxy derivatives of AA, EPA, and DHA differs from that of other fish tissues. For example, trout leucocytes, although displaying a prominent 12-lipoxygenase activity and DHA in their phospholipids, do not yield appreciable amounts of 14-HDHE upon calcium ionophore challenge (6,7). This may suggest some diversity in the 12-lipoxygenase activity of gill and other tissues, such as blood. A similar situation is also apparent in

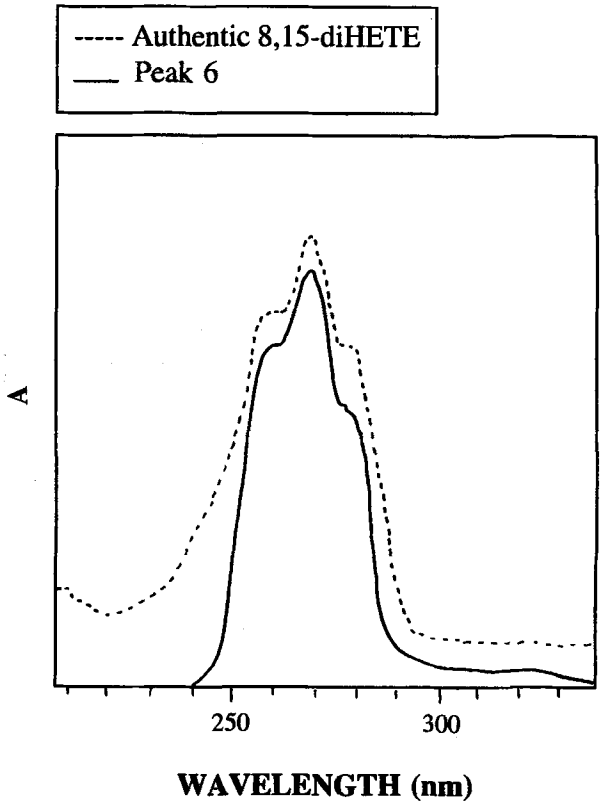


FIG. 5. On-line ultraviolet scans of authentic 8(*R*),15(*S*)-diHETE and material under peak 6 both separated by reverse-phase high-performance liquid chromatography. These have a λmax of 268 nm and shoulders at *ca.* 258 and 278 nm. Abbreviation as in Figure 3.

mammals with "platelet" and "leucocyte" types of 12-lipoxygenase (28).

Although the identification of 14-HDHE in trout gills is tentative in the absence of commercially available standards, previous studies using gas chromatography/mass spectrometry have verified its presence in this tissue (5). More recently, Bell *et al.* (12) have identified this same compound in Atlantic salmon (*Salmo salar*) gills based on relative retention times during RP-HPLC and ^{14}C DHA incubation. None of these reports, including the present study on fish gills, have shown the generation of 11-HDHE as found in human platelets following incubation with exogenous DHA (29). Aveldaño and Sprecher (29) hypothesized that as the ratio of 14-HDHE to 11-HDHE generated by these platelets varied depending on the concentration of exogenous fatty acid precursor, implying the presence of two forms of lipoxygenase with different affinities for DHA. Incubation of trout gill in the present study with varying concentrations of DHA did not result in the generation of any additional peaks in the region of the chromatograph of the other HETEs/HEPEs that could have been 11-HDHE.

Previous studies have revealed the propensity of fish leucocytes to yield LXs (7,24) and that the biosynthesis of LXA_4 in trout macrophages occurs by the synthetic collaboration of 5- and 12-lipoxygenase activity found in this cell type (30). The only tissues/organs that generated LXs in the present study were the ovary, spleen, and brain (Table 1). It is possible that this biosynthesis was due to the contamination of these organs with leucocytes; however, other highly vascularized organs such as gills, liver, and heart were found to lack the ability to generate LXs at levels above the detection limit of RP-HPLC. As microglial cells are derived from monocytes, these cells are potential candidates as the source of LXs in trout brain. Therefore, immunocytochemical studies were carried out using monoclonal antibodies raised against trout macrophages that also react with all of the leucocyte types. These antibodies failed to demonstrate any immunoreactive microglial cells in all of the regions of brain examined. In another species of fish, the tilapia (*Oreochromis mossambicus*), abundant macrophages and lymphocytes that react with an anti-CD45-like monoclonal antibody have been found in the central nervous system particularly in myelinated fiber tracts (22), suggesting the potential for large populations of leucocytes with eicosanoid-generating ability in the central nervous system of fish. Furthermore, astroglial cells from the rainbow trout, *O. mykiss*, generate a range of 5- and 12-lipoxygenase products upon calcium ionophore challenge, including $LTB_{4/5}$, 12-HETE, and 12-HEPE (31), although the capacity of these cells to synthesize lipoxins was not determined. Finally, it is pertinent to note that LX generation has also been reported in mammalian brain (32,33) and that ischemia (32) and HIV infection (33) enhance the generation of these compounds.

Rainbow trout gill displayed a different profile of di-HETE/di-HEPE generation than the other tissues examined. Although a peak was found in RP-HPLC chromatograms that co-eluted with authentic LTB_4 in two solvent systems, specific inhibitors failed to modify the production of this material, implying that this compound was not present. Caution should be exercised in such a conclusion, however, as the specificity and efficacy of these inhibitors have not been reported in fish. Furthermore, initial studies with the LTA hydrolase inhibitor, captopril, used in the present study, have shown that the biosynthesis of lipoxygenase products other than $LTB_{4/5}$ in trout leucocytes is also inhibited (Lloyd-Evans, P., unpublished observations), perhaps suggesting a lack of selectivity in the action of this compound. Other studies with the gills from rainbow trout (11) and Atlantic salmon (12) have failed to detect $LTB_{4/5}$ generation although LTB_4 has been identified as a major lipoxygenase product in killifish (*Fundulus heteroclitus*) gills (34). It should be pointed out, however, that this identification of LTB_4 in killifish gills was only based on RP-HPLC and detection of ^{14}C AA-labelled products without co-chromatography with authentic standards or mass spectrometry. The limited generation of material that co-chromatographs with authentic LTB_4 in the present study and its proximity to other material in chromatograms (e.g., peak 8 in Fig. 3) would also make it difficult to confirm the identity of the compound(s) produced using gas chromatography/mass spectrometry. What is clear, however, is that even if LTB_4 and LTB_5 are generated by rain-

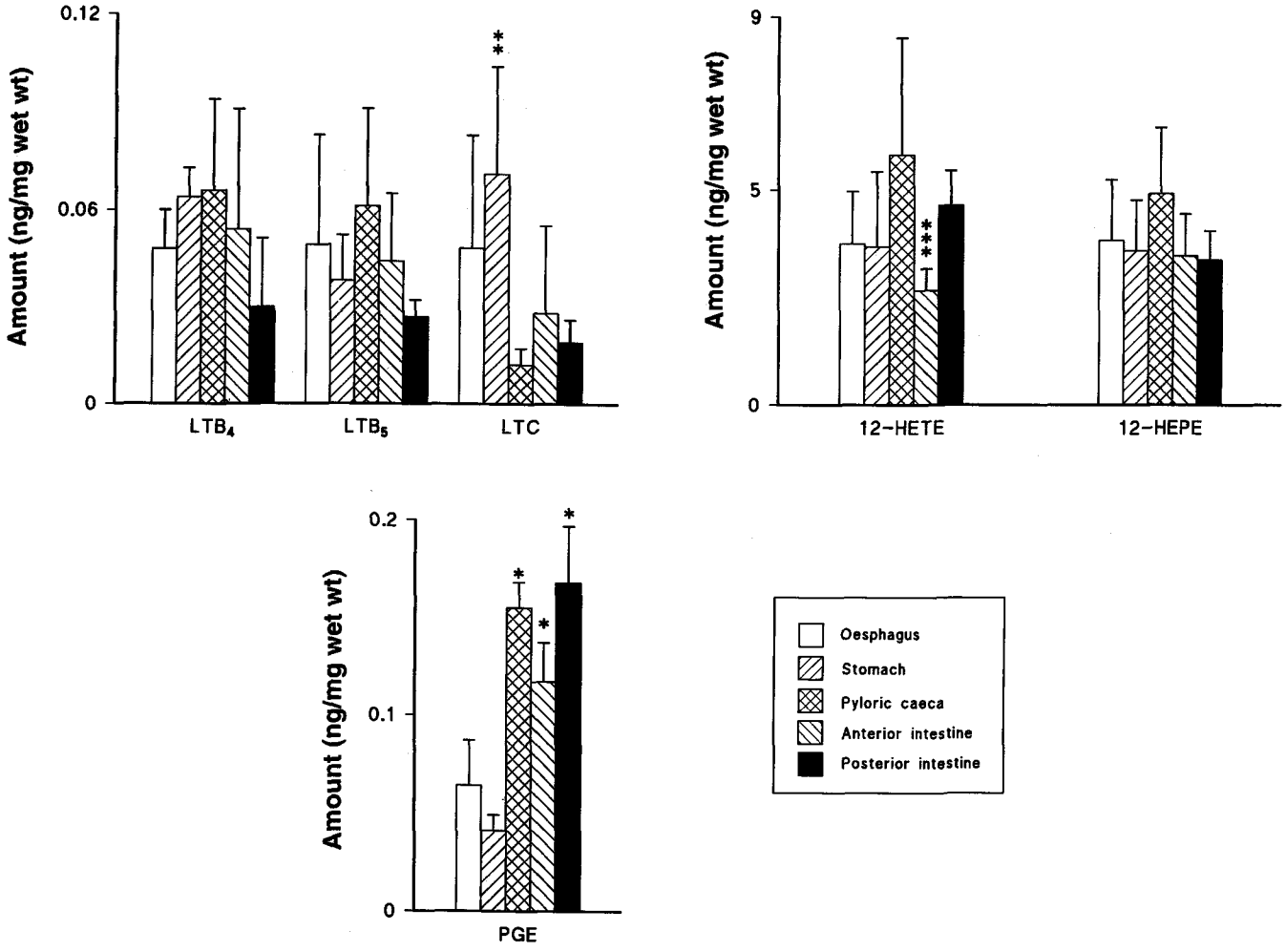


FIG. 6. Generation of various eicosanoids by different regions of the alimentary canal of the rainbow trout, *Oncorhynchus mykiss*. Tissues were incubated with 5 μM calcium ionophore, A23187, for 10 min at 18°C, and the reaction was terminated by centrifugation. Supernatants were used without extraction for enzyme immunoassay analysis of LTC and PGE levels, and for reverse-phase high-performance liquid chromatography, samples were Sep-Pak extracted following the addition of PGB_2 (100 ng) as an internal standard. Values are means ± SD, n = 3–5. $^*P < 0.005$ compared with values for esophagus and stomach, $^{**}P < 0.05$ compared with values for pyloric caeca and posterior intestine, and $^{***}P < 0.05$ compared with values for the posterior intestine. Abbreviations as in Figures 1 and 3.

bow trout gill, they are very minor components and are therefore unlikely to be of physiological consequence.

The gills of rainbow trout do generate appreciable amounts of LTC-immunoreactive material, implying the presence of 5-lipoxygenase and LTC synthase activity in this tissue. Furthermore, LTC_4, LTD_4, and LTE_4 have been convincingly identified in gills from the eel, *Anguilla rostata* (13). Other 5-lipoxygenase products, such as 5(*S*)-HETE, were not observed in ionophore-challenged supernatants of trout gill in the present study and, similarly, other trout tissues that have been shown to contain 5-lipoxygenase activity, including blood, do not synthesize appreciable amounts of 5-HETE in comparison to 12-HETE and 12-HEPE (6,7). The only di-HETE tentatively identified in the present study by RP-HPLC was 8(*R*),15(*S*)-diHETE, a dual 12- and 15-lipoxygenase product previously observed in crude gill homogenates of trout as the major dihydroxy metabolite of exogenous AA (11). Its potential finding in intact gill material from endogenous stores of AA adds weight to the suggestion of German and Berger (11) that it may be of physiological importance. Clearly, a large number of potential mono- and di-hydroxy metabolites of AA and EPA can be produced by fish gills as a result of the 5-,12- and 15-lipoxygenase activities present. It should be of interest to examine the biological significance of some of these lipoxygenase metabolites in fish gills as both cyclooxygenase and lipoxygenase products, including PGE_2, LTC_4, LTD_4, and LTE_4 appear to play a role in the regulation of ion transport in the gills of the killifish, *F. heteroclitus* (34).

ACKNOWLEDGMENTS

J.K. was supported by a SERC earmarked studentship awarded to A.F.R., while J.H. and L.A.B. were supported by NERC studentships. K.H. was a recipient of a Wellcome Trust Vacation Studentship during the period of these experiments. MK-886 was kindly provided by the Merck-Frosst Centre for Therapeutic Research, Quebec, Canada.

REFERENCES

1. Samuelsson, B., Dahlén, S.-E., Lindgren, J.Å., Rouzer, C.A., and Serhan, C.N. (1987) *Science (N.Y.) 237*, 1171–1175.
2. Phipps, R.P., Stein, S.H., and Roper, R.L. (1991) *Immunol. Today 12*, 349–352.
3. Moore, P.K. (1986) *Prostanoids: Pharmacological, Physiological and Clinical Relevance*, Cambridge University Press, Cambridge.
4. Henderson, R.J., and Sargent, J.R. (1986) in *Nutrition and Feeding in Fish* (Cowey, C.B., Mackie, A.M., and Bell, J.G., eds.) pp. 349–364, Academic Press, London.
5. German, J.B., Bruckner, G.G., and Kinsella, J.E. (1986) *Biochim. Biophys. Acta 875*, 12–20.
6. Pettitt, T.R., Rowley, A.F., and Barrow, S.E. (1989) *Biochim. Biophys Acta 1003*, 1–8.
7. Pettitt, T.R., Rowley, A.F., Barrow, S.E., Mallet, A.I., and Secombes, C.J. (1991) *J. Biol. Chem. 266*, 8720–8726.
8. Matsumoto, H., Iijima, N., and Kayama, M. (1989) *Comp. Biochem. Physiol. 93B*, 397–402.
9. Rowley, A.F. (1991) *Biochim. Biophys. Acta 1084*, 303–306.
10. Tocher, D.R., and Sargent, J.R. (1985) *Comp. Biochem. Physiol. 87B*, 733–739.
11. German, J.B., and Berger, R. (1990) *Lipids 25*, 849–853.
12. Bell, J.R., Sargent, J.R., and Raynard, R.S. (1992) *Prost. Leukotr. Essential Fatty Acids 45*, 197–206.
13. Piomelli, D. (1985) *Naturwissenschaften 72*, 276–277.
14. Beckman, B., and Mustafa, T. (1992) *Fish Physiol. Biochem. 10*, 213–222.
15. Mohri, S., Cho, S-Y., Endo, Y., and Fujimoto, K. (1992) *J. Agric. Food Chem. 40*, 573–576.
16. Goetz, F.W. (1991) *Amer. J. Physiol. 260*, R862–R865.
17. Wade, M.G., and van der Kraak, G. (1993) *J. Exp. Zool. 266*, 108–115.
18. Hsu, S.-Y., and Goetz, F.W. (1993) *Biol. Reprod. 48*, 1202–1209.
19. Mustafa, T., Agnisola, C., and Tota, B. (1992) *Comp. Biochem. Physiol. 103C*, 163–167.
20. Lowenstein, J. (1991) *Comp. Biochem. Physiol. 98B*, 389–396.
21. Perry, V.H., and Lawson, L.J. (1992) in *The Macrophage* (Lewis, C.E., and McGee, J.O'D., eds.) pp. 391–413, IRL Press, Oxford.
22. Dowding, A.J., and Scholes, J. (1993) *Proc. Natl. Acad. Sci. USA 90*, 10183–10187.
23. Hertting, G., and Seregi, A. (1989) *Annals N.Y. Acad. Sci. 559*, 84–99.
24. Lloyd-Evans, P., Barrow, S.E., Hill, D.J., Bowden, L.A., Rainger, G.E., Knight, J., and Rowley, A.F. (1994) *Biochim. Biophys. Acta 1215*, 291–299.
25. Galfre, G., Howe, S.C., Milstein, C., Butcher, G.W., and Howard, J.C. (1977) *Nature 266*, 550–552.
26. Serhan, C.N. (1994) *Biochim. Biophys. Acta 1212*, 1–25.
27. Orning, L., Krivi, G., Bild, G., Gierse, J., Aykent,S., and Fitzpatrick, F.A. (1991) *J. Biol. Chem. 266*, 16507–16511.
28. Yamamoto, S. (1992) *Biochim. Biophys. Acta 1128*, 117–131.
29. Aveldaño, M.I., and Sprecher, H. (1983) *J. Biol. Chem. 258*, 9339–9343.
30. Rowley, A.F., Lloyd-Evans, P., Barrow, S.E., and Serhan, C.N. (1994) *Biochemistry 33*, 856–863.
31. Tocher, D.R., Bell, J.G., and Sargent, J.R. (1991) *J. Neurochem. 57*, 2078–2085.
32. Kim, S.J., and Tominaga, T. (1989) *Annals. N.Y. Acad. Sci. 559*, 461–464.
33. Genis, P., Jett, M., Bernton, H.W., Boyle, T., Gelbard, H.A., Dzenko, K., Keane, R.W., Resnick, L., Mizrachi, Y., Volsky, D.J., Epstein, L.G., and Gendelman, H.E. (1992) *J. Exp. Med. 176*, 1703–1718.
34. Van Praag, D., Farker, S.J., Minkin, E., and Primor, N. (1987) *Gen. Comp. Endocrinol. 67*, 50–57.

[Received November 18, 1994; and in revised form February 10, 1995; Revision accepted March 7, 1995]

New Branched-Chain Fatty Acids from the Senegalese Gorgonian *Leptogorgia piccola* (white and yellow morphs)

Joseph Mirallès[a], Gilles Barnathan[b], Renée Galonnier[c], Thioro Sall[c], Abdoulaye Samb[c], Emile Marcel Gaydou[d] and Jean-Michel Kornprobst[b,*]

[a]Département de Biologie Végétale, Faculté des Sciences, Université Cheikh Anta Diop, Dakar, Sénégal, [b]Groupe SMAB, Faculté de Pharmacie, Université de Nantes, 44035 Nantes Cedex 01, France, [c]Département de Chimie, UCAD, Dakar, Sénégal and [d]Laboratoire de Phytochimie, Faculté des Sciences et Techniques de Saint-Jérôme, Université d'Aix-Marselle III, Marseille, France

ABSTRACT: Fatty acids from total lipids of the gorgonian *Leptogorgia piccola* (white and yellow morphs), collected from the same area at two different periods with regard to the average water temperature, were studied. More than fifty fatty acids were identified as methyl esters and *N*-acyl pyrrolidides by gas chromatography and gas chromatography/mass spectrometry. Three new, branched-chain unsaturated fatty acids were identified in addition to the unusual 7-methyl-6-hexadecenoic acid, namely 10-methyl-6-hexadecenoic, 7,9-dimethyl-6-hexadecenoic, and 10-methyl-6,9-heptadecadienoic acids. Also 6,9-heptadecadienoic acid was identified. The fatty acid patterns of specimens harvested in colder waters were quite different from those harvested in warmer waters in that the former contained high amounts of methylene-interrupted polyunsaturated acids, including tetracosapolyenoic acids, especially 6,9,12,15,18-24:5 (up to 15.8% of the total acid mixture) and 6,9,12,15,18,21-24:6 (up to 5.3%). Arachidonic acid was, nevertheless, a major component in all the fatty acid mixtures studied (13.6–20.5%). Based on gas chromatography/Fourier transform infrared experiments, the double bonds were assigned the (*Z*) configuration. Several fatty aldehydes and their dimethyl acetals were also detected, of which the most abundant was octadecanal.
Lipids 30, 459–466 (1995).

Marine invertebrates, such as sponges and gorgonians, are known to contain many unusual metabolites, especially terpenoids, sterols, and fatty acids. We recently described the sterol composition of nine Senegalese gorgonians (Cnidaria, Octocorallia) including *Leptogorgia piccola* (1), which also contained four novel terpenoid secondary metabolites (2). The fatty acid composition of several classes of marine invertebrates, including Cnidaria, have been reviewed (3–5). The fatty acid composition of an unidentified sublittoral Caribbean gorgonian was reported first, revealing low amounts of polyunsaturated fatty acids (PUFAs) (6). Fatty acids from some Octocorallia species have also been identified, including gorgonians *Plumarella longispina* and *Paragorgia arborea*, which contained high amounts of 6,9,12,15,18,21-24:6 and 6,9,12,15,18-24:5 acids (7). Eicosapentaenoic acid (5,8,11,14,17-20:5) was the major component of the former organism, and 5,8,11,14-20:4 (arachidonic acid) was that of the latter. These icosapolyenoic acids are known to possess remarkable pharmacological effects in higher animals. They are membrane phospholipid components and important prostaglandin precursors. An interesting finding was the occurrence of arachidonic acid as a major fatty acid (>20%) in lipids of the gorgonian *Plexaura homomalla* (8), which also contains some prostaglandin derivatives accounting for 1.8% of its dry weight (9).

We have investigated gorgonians as part of our continuing research on lipids from Cnidaria (10). This report details the fatty acid composition of total lipids from *L. piccola*. These Senegalese gorgonians were encountered as two color morphs (white and yellow) in the same area, but mixed colonies have never been observed (11). As a first step in this study, lipids from specimens harvested during the cool season (November to April) were investigated. Fatty acids were determined by their gas chromatography (GC) retention behavior on either nonpolar or polar columns (by comparison with commercially available standards) and by GC/mass spectrometry (GC/MS) studies for the most prominent GC peaks. In order to investigate whether water temperature had effects on fatty acid composition, additional specimens of *L. piccola*, collected at the beginning of the hot season (June), were studied. In Senegal, the cool season corresponds to a period of important upwelling characterized by cold and saline water (averaging 16–20°C). The hot season usually begins with a rapid warming of seawater (more than 25°C) (12,13). These latter samples were studied in more detail, using GC/MS to elucidate the structure of several unusual minor fatty acids encountered in colder water samples. Surprising differences between the two harvests were observed concerning relative abundance of dif-

*To whom correspondence should be addressed at Groupe SMAB, Faculté de Pharmacie, 1, rue Gaston Veil, BP 1024, 44035 Nantes Cedex 01, France.

Abbreviations: ECL, equivalent chain length; FAME, fatty acid methyl esters; GC, gas chromatography; GC/FTIR, gas chromatography/Fourier transform infrared; GC/MS, gas chromatography/mass spectrometry; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

ferent PUFA. Several interesting branched short-chain unsaturated fatty acids were identified and are described in this paper.

EXPERIMENTAL PROCEDURES

Gorgonian material and lipid extraction. Both morphs (white and yellow) of *L. piccola* were collected by hand (scuba diving) 20 km off Dakar (Senegal) at an average depth of 30 m, first from November 1989 to April 1990 (colder water) and then in June 1992 (warm water). Total lipids of the colder water gorgonian samples were extracted by the Bligh and Dyer method (14). The chloroform layer was filtered and evaporated under vacuum at room temperature, yielding a lipid residue which was saponified with 2N ethanolic potassium hydroxide. The unsaponifiable matter was separated, and the remaining aqueous fraction acidified. Fatty acids were recovered by *n*-hexane extractions and converted into methyl esters using BF_3/methanol (15). Crude fatty acid methyl esters (FAME) were purified by thin-layer chromatography (TLC) on Kieselgel 60 0.5-mm thick precoated plates (Macherey-Nagel, Doren, Germany) by development with benzene/methanol (98:2, vol/vol). The gorgonian samples from warmer water were ground in a Waring blender (Bioblock-Scientific, Strasbourg-Illkirch, France) and steeped twice in dichloromethane/methanol (1:1, vol/vol) for 24 h (room temperature). Combined crude total lipids were saponified as above and converted into FAME by refluxing with 2% methanolic hydrogen chloride (40 min). To determine double-bond positions and methyl branching, FAME were converted into *N*-acyl pyrrolidides by treatment with pyrrolidine/acetic acid (10:1, vol/vol) under reflux (2 h). Pyrrolidides were purified by TLC on 0.5-mm silica gel layers using hexane/diethyl ether (1:2, vol/vol) as developing solvent. FAME mixtures were hydrogenated by stirring (4 h) at ambient pressure and temperature in methanol in the presence of catalytic amounts of platinum (IV) oxide (Adam's catalyst). Many of the fatty acids reported here were simply identified by comparing their GC mobilities as methyl esters with those of known natural mixtures (salmon oil lipids) and by co-injection with commercial standards. Capillary GC analysis of a hydrogenated aliquot also provided useful information for detection and identification of unsaturated acids.

Gas–liquid chromatography. Analytical GC was carried out on a Carlo-Erba 4130 apparatus (Milano, Italy) with a flame-ionization detector linked to a Spectra Physics model 4270 numerical integrator (St. Albans, United Kingdom) for data collection. GC analyses were performed on two types of fused silica capillary columns: a polar Carbowax-20M (50 m × 0.32 mm i.d., 0.40 μm phase thickness) and a nonpolar OV-1 (25 m × 0.32 mm i.d., 0.40 μm phase thickness) (A.M.L.-chromato, Limoges, France). Oven temperature was 195°C for the former column and 180°C for the latter. Common FAME were identified by comparison of their retention behavior on either column (equivalent chain length, ECL) with those of authentic standards (Sigma Chemical Co., St. Louis, MO) or salmon oil FAME that had been studied previously (16), and confirmed by GC/MS experiments.

GC/MS. For the gorgonian specimens harvested from November to April, combined GC/MS was performed on a Girdel 30 gas chromatograph linked to a Ribermag R-10-10B mass spectrometer and coupled with a Sidar computer. The GC column was a 25 m × 0.32 mm i.d. fused silica capillary column coated with OV-1 (0.40 μm phase thickness). FAME were analyzed from 180 to 300°C at 3°C/min and pyrrolidide derivatives from 220 to 300°C at 3°C/min (carrier gas, helium; ion source, 180°C; ionizing voltage, 70eV). For the gorgonian specimens harvested in June, combined GC/MS was performed on a Hewlett-Packard HP-5890 chromatograph linked to an HP 9000/345 integrator (Palo Alto, CA). The GC column was a 30 m × 0.32 mm i.d. fused silica capillary column coated with DB-1 (0.25 μm phase thickness). The carrier gas was helium. Column temperature was programmed from 180 to 310°C, at 3°C/min, for both derivatives (FAME and pyrrolidides).

Gas chromatography/Fourier transform infrared (GC/FTIR). For specimens harvested in June, GC/FTIR spectra of the FAME were recorded with a 20S×B Nicolet spectrometer (Madison, WI) interfaced with a 5300 Mega Carlo Erba FID chromatograph equipped with a fused silica capillary (DB-1, 60 m × 0.32 mm i.d., 0.25 μm phase thickness; J&W Scientific, Folson, CA). Oven temperature was programmed from 70 to 130°C (15°C/min) and from 130 to 290°C (4°C/min). The carrier gas was helium. Light-pipe temperature was maintained at 293°C.

RESULTS

As determined by the Bligh and Dyer procedure for the colder water specimens (dry weight), the white morph yielded 2.1% of total lipids, and the yellow morph (dry weight) yielded 1.9%. As determined by the dichloromethane/methanol extraction procedure for the warmer water specimens (dry weight), the white morph yielded 8.4% of total lipids, and the yellow morph yielded 4.9%.

The fatty acid composition of the two morphs of *L. piccola* (at two different sampling periods) is given in Table 1. More than fifty fatty acids were identified in gorgonian lipids, most being present in the four samples studied.

Colder water specimens contained 21.5% (white morph) and 26.9% (yellow morph) of saturated fatty acids, whereas warmer water specimens contained 51.3 and 53.4%, respectively. On the contrary, colder water specimens contained 66.3% (white morph) and 55.9% (yellow morph) of PUFAs, whereas warmer water specimens contained 25.3 and 29.1, respectively. The partial *m/z* 113 ion monitoring GC/MS chromatogram of fatty acid pyrrolidides is presented in Figure 1.

Mass spectral data of selected fatty acid pyrrolidides are as follows: *10-Methyl-6-hexadecenoic acid pyrrolidide* **4**. MS *m/z* (rel. intensity): 321 (M^+, 1.3), 306 (0.3), 292 (0.1), 278 (0.4), 264 (0.3), 250 (0.5), 236 (1.1), 222 (0.3), 208 (4.2), 196

TABLE 1
Total Lipid Fatty Acids from the Gorgonian *Leptogorgia piccola* (white and yellow morphs) Including Colder Water Specimens (November 1989 to April 1990) and Warmer Water Specimens (June 1992)

	White morph %		Yellow morph %	
Fatty acids	Colder water specimens	Warmer water specimens	Colder water specimens	Warmer water specimens
Saturated Fatty acids				
12:0	—[a]	0.3	trace[b]	0.7
14:0	1.4	5.6	2.0	4.4
4,8,12-Trimethyl-13:0	0.3	0.4	0.3	0.4
11-Methyl-14:0	—	—	—	0.7
i-15:0[c]	trace	1.0	trace	—
15:0	0.4	2.2	0.5	1.8
16:0	9.7	15.8	13.0	14.3
13-Methyl-16:0	—	—	—	0.6
i-17:0	0.5	2.2	0.7	1.8
ai-17:0[d]	0.4	—	trace	1.0
17:0	0.7	5.0	1.2	5.2
3,7,11,15-Tetramethyl-16:0	trace	0.6	trace	0.5
18:0	7.0	12.6	8.0	16.7
i-19:0	—	0.7	—	0.9
19:0	trace	trace	trace	1.6
20:0	0.7	3.4	0.5	2.0
22:0	0.4	0.6	0.7	trace
24:0	trace	0.9	trace	0.8
Total %	21.5	51.3	26.9	53.4
Monoenoic fatty acids				
6-16:1	}4.8	1.1	}5.8	1.1
8-16:1		5.7		2.3
10-Methyl-6-16:1[e]	n.i.[f]	0.7	n.i.	0.7
7-Methyl-6-16:1	0.7	1.4	1.8	3.7
8-17:1	n.i.	1.1	n.i.	—
7,9-Dimethyl-6-16:1[e]	0.3	0.4	0.9	0.9
9-18:1	2.5	3.3	3.8	3.7
11-18:1	3.2	4.4	4.4	2.3
6-20:1	—	0.6	—	—
11-20:1	trace	0.6	trace	0.4
13-20:1	—	0.8	—	0.3
6-21:1	—	0.5	—	—
11-22:1	trace	0.4	trace	0.4
15-24:1	trace	0.7	trace	0.4
Total %	11.5	21.7	16.7	16.2
Polyunsaturated fatty acids				
6,9-17:2	—	trace	—	0.8
10-Methyl-6,9-17:2[e]	n.i.	0.3	n.i.	0.3
6,9,12-18:3	0.6	0.6	trace	0.3
9,12,15-18:3	0.6	0.4	1.0	0.5
9,12-18:2	1.0	1.8	1.3	3.7
5,8,11,14,17-20:5	8.0	0.6	8.1	0.6
5,8,11,14-20:4	20.5	14.7	18.9	13.6
8,11,14,17-20:4	trace	2.3	trace	4.0
4,7,10,13,16,19-22:6	3.8	trace	4.1	—
4,7,10,13,16-22:5	1.5	1.1	0.7	0.8

(continued)

TABLE 1 (continued)

Fatty acids	White morph %		Yellow morph %	
	Colder water specimens	Warmer water specimens	Colder water specimens	Warmer water specimens
7,10,13,16,19-22:5	1.2	}0.4	1.2	}1.0
7,10,13,16-22:4	2.8		2.2	
6,9,12,15,18,21-24:6	5.3	trace	5.0	trace
6,9,12,15,18-24:5	15.8	2.1	8.4	2.4
9,12,15,18,21-24:5	5.2	0.4	5.0	1.2
9,12,15,18-24:4	trace	0.6	trace	0.7
Total %	66.3	25.3	55.9	29.1

[a]– <0.1%.

[b]Minor fatty acids (<0.3%) 8:0, 9:0, 10:0, 13:0, *ai* 15:0, 13-18:1, 11,14,17-20:3, 11,14-20:2, 15-22:1.

[c]*i*, iso.

[d]*ai*, anteiso.

[e]Not previously described in nature.

[f]n.i., Not identified.

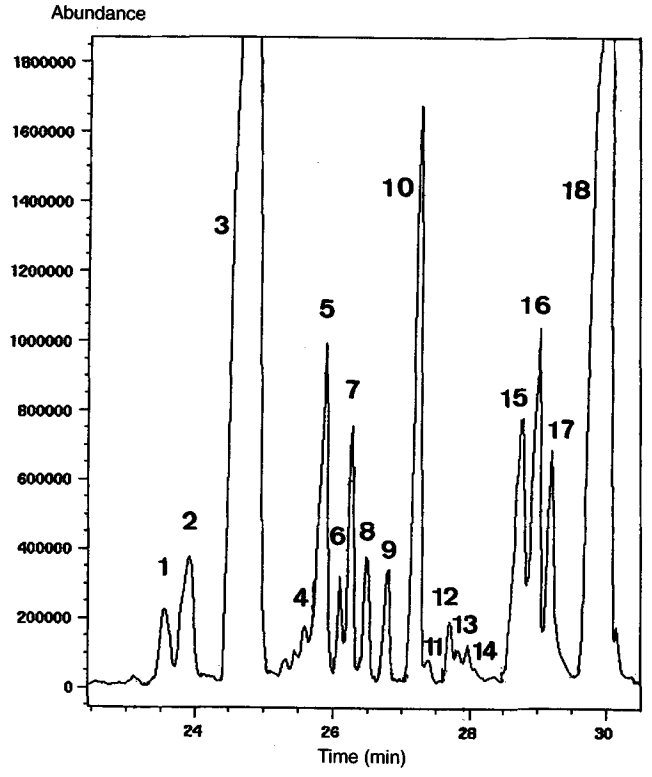


FIG. 1. Ion monitoring *m/z* 113 of fatty acid pyrrolidides from *Leptogorgia piccola* (yellow morph) from warmer water specimens. **1**, 6-Hexadecenoic; **2**, 8-hexadecenoic; **3**, hexadecanoic; **4**, 10-methyl-6-hexadecenoic; **5**, 7-methyl-6-hexadecenoic; **6**, 6,9-heptadecadienoic; **7**, 13-methylhexadecanoic; **8**, 14-methylhexadecanoic; **9**, 7,9-dimethyl-6-hexadecenoic; **10**, heptadecanoic; **11**, 10-methyl-6,9-heptadecadienoic; **12**, 9,12,15-octadecatrienoic; **13**, phytanic; **14**, 6,9,12-octadecatrienoic; **15**, 9,12-octadecadienoic; **16**, 9-octadecenoic; **17**, 11-octadecenoic; **18**, octadecanoic.

(2.0), 194 (2.4), 182 (0.9), 180 (2.2), 168 (1.8), 166 (4.5), 165 (2.4), 154 (1.7), 140 (2.2), 126 (20.6), 113 (100), 98 (7.7), 85 (7.5), 72 (10.6), 70 (10.4).

7-Methyl-6-hexadecenoic acid pyrrolidide **5**. MS *m/z* (rel. intensity): 321 (M^+, 6.3), 306 (0.5), 292 (0.6), 278 (0.6), 264 (0.5), 250 (0.6), 236 (1.5), 222 (1.7), 208 (7.4), 194 (3.0), 180 (0.2), 166 (6.0), 154 (4.3), 140 (4.9), 127 (11.3), 126 (35.4), 113 (100), 98 (10.7), 85 (3.1), 72 (21.5), 70 (14.0).

7,9-Dimethyl-6-hexadecenoic acid pyrrolidide **9**. MS *m/z* (rel. intensity): 335 (M^+, 8.2), 320 (0.7), 306 (0.3), 292 (0.3), 278 (07), 264 (0.8), 250 (1.8), 236 (3.6), 222 (0.2), 210 (1.7), 209 (5.3), 208 (38.5), 196 (2.8), 195 (1.5), 194 (2.3), 182 (0.5), 180 (0.8), 168 (5.9), 166 (6.5), 154 (3.8), 140 (5.9), 126 (36.2), 113 (100), 98 (16.9), 85 (3.1), 72 (30.8), 70 (14.6).

6,9-Heptadecadienoic acid pyrrolidide **6**. MS *m/z* (rel. intensity): 319 (M^+, 1.5), 305 (10.0), 276 (<1.0), 262 (<1.0), 248 (1.5), 234 (2.3), 220 (2.4), 208 (1.8), 206 (2.0), 194 (2.1), 180 (2.5), 168 (4.6), 166 (4.9), 154 (4.5), 140 (4.7), 126 (32.3), 113 (100), 98 (16.9), 85 (16.3), 72 (34.9), 70 (35.3).

10-Methyl-6,9-heptadecadienoic acid pyrrolidide **11.** MS *m/z* (rel. intensity): 333 (M^+, 3.2), 318 (0.7), 304 (0.3), 290 (0.2), 276 (0.2), 262 (0.3), 248 (0.4), 234 (1.6), 220 (0.1), 210 (0.7), 206 (1.2), 194 (0.7), 180 (6.2), 167 (3.9), 166 (2.4), 154 (1.5), 140 (1.6), 126 (6.2), 113 (100), 98 (10.8), 85 (4.5), 72 (5.2), 71 (4.3), 70 (4.3).

3,7,11,15-Tetramethylhexadecanoic acid (phytanic acid) pyrrolidide. MS *m/z* (rel. intensity): 365 (M^+, 0.7), no peak at *m/z* 366, 350 (1.6), 322 (0.5), 308 (0.3), 294 (0.5), 280 (0.4), 266 (0.1), 252 (0.7), 238 (0.2), 224 (0.9), 210 (1.0), 196 (0.4), 194 (0.3), 182 (2.5), 180 (20.), 168 (0.9), 166 (0.6), 154 (2.3), 140 (12.3), 126 (4.1), 113 (100), 98 (13.8), 85 (5.4), 72 (12.1), 71 (12.5), 70 (12.3), 57 (10.7), 55 (26.2), 43 (26.2), 41 (12.2), 29 (4.6).

Saturated fatty acids. Two common isoprenoid fatty acids were detected in the total lipids as minor components, namely 4,8,12-trimethyltridecanoic and 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid). They were readily identified from GC and coupled GC/MS data concerning their methyl esters (Table 2). GC/MS data of pyrrolidide derivatives con-

TABLE 2
Equivalent Chain Length (ECL) Values of Selected Branched-Chain Fatty Acids of the Gorgonian *Leptogorgia piccola* (yellow morph)

Fatty acids	ECL (DB-1)	
	Methyl esters	Pyrrolidides[a]
4,8,12-Trimethyl-13:0	14.53	14.32
11-Methyl-14:0	14.63	14.62
10-Methyl-6-16:1	16.37	16.35 (**4**)
7-Methyl-6-16:1	16.47	16.46 (**5**)
13-Methyl-16:0	16.64	16.63 (**7**)
7,9-Dimethyl-6-16:1	16.83	16.82 (**9**)
10-Methyl-6,9-17:2	17.06	17.06 (**11**)
3,7,11,15-Tetramethyl-16:0	17.72	17.23 (**13**)

[a]Numbers in boldface refer to gas chromatography/mass spectrometry chromatogram (Fig. 1).

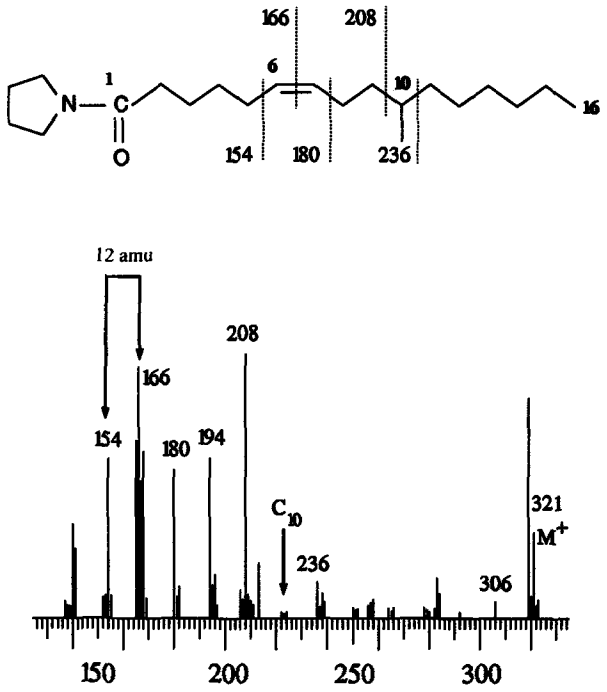


FIG. 2. Partial mass spectrum of 10-methyl-6-hexadecenoic acid pyrrolidide **4** (×7 expanded).

firmed the structures. The mass spectrum of the pyrrolidide of phytanic acid is described in the Results section, which to our knowledge has not been previously reported. Furthermore, the GC behavior of these isoprenoid acid pyrrolidides appeared noteworthy. As shown in Table 2, the phytanic acid pyrrolidide (**13** in Fig. 1) eluted faster than the corresponding methyl ester (ECL of 17.23 and 17.72, respectively), all things being equal.

This effect seems convenient for GC analyses as the phytanic acid methyl ester usually falls in a rather crowded area just before stearic acid methyl ester. 4,8,12-Trimethyltridecanoic acid pyrrolidide presented similar behavior, but to a lesser extent (ECL of 14.32 instead of 14.53 for the methyl ester; Table 2). Another interesting result was the occurrence in only one gorgonian sample (yellow morph harvested in June) of 11-methyltetradecanoic and 13-methylhexadecanoic **7** acids (Table 1), which were readily identified by GC/MS of their pyrrolidides. These unusual fatty acids previously have not been described in gorgonian lipids.

Monoenoic fatty acids. Four interesting unsaturated fatty acids, the first three being monounsaturated, were detected as methyl esters and characterized as pyrrolidide derivatives (**4,5,9,11**). The first acid in question (the methyl ester) showed a molecular ion peak at *m/z* 282, corresponding to a 17:1 acid structure, and an ECL value of 16.35, implying methyl branching. Upon catalytic hydrogenation, the compound was converted into the methyl ester of 10-methylhexadecanoic acid (ECL 16.42) occurring in marine sponges as a natural product, probably of bacterial origin (17,18). The position of the double bonds and the branching were determined from GC/MS data for pyrrolidide derivative **4** (Fig. 2).

The molecular ion peak was present at *m/z* 321, confirming a 17:1 structure. The difference of 12 amu betweeh homologous fragmentation ions at *m/z* 154 (C_5) and *m/z* 166 (C_6) indicated Δ6 unsaturation. A very diminished peak at *m/z* 222 (C_{10}), flanked by an elevated peak at *m/z* 208 (C_9), and an appreciable peak at *m/z* 236 indicated a methyl branch at C-10. Thus, we identified the new 10-methyl-6-hexadecenoic acid, which to our knowledge, has not been reported in any natural source.

The second interesting short-chain fatty acid (the methyl ester) showed a molecular ion peak at *m/z* 282 corresponding to a 17:1 acid structure and an ECL value of 16.46, also implying methyl branching. The GC peak disappeared upon catalytic hydrogenation. This fatty acid was readily identified as the already known, but unusual, 7-methyl-6-hexadecenoic acid (19,20) as its pyrrolidide **5** (molecular ion peak at *m/z* 321) showed a difference of 12 amu between fragmentation ions *m/z* 154 (C_5) and 166 (C_6) (Δ6 unsaturation), and a very low peak at *m/z* 180 (C_7) flanked by two elevated peaks at *m/z* 166 and 194 (C_8) (7-methyl branch).

The third fatty acid (the methyl ester) showed a very low ECL value of 16.82 for a molecular ion peak at *m/z* 296, indicating a 18:1 acid structure. These data clearly suggested methyl branching. The peak corresponding to this methyl ester disappeared upon catalytic hydrogenation. The corresponding pyrrolidide derivative **9** showed a molecular ion peak at *m/z* 335, confirming the 18:1 acid structure (Fig. 3). Δ6 Unsaturation was proved by a difference of 12 amu between homologous fragmentation ions at *m/z* 154 and 166. A diminished peak at *m/z* 180 (C_7), flanked by an intense peak at *m/z* 166 (C_6), and an appreciable peak at *m/z* 194 (C_8), indicated a methyl branch at C-7. A second methyl branch occurred at C-9 position as the expected peak at *m/z* 222 (C_{10} fragment but C-9 position) appeared with a very low relative intensity between two elevated peaks at *m/z* 208 (C_9 fragment but C-8 position) and *m/z* 236 (C_{11} fragment but C-10 position). Thus, we identified the 7,9-dimethyl-6-hexadecenoic acid, not yet reported in nature.

Dienoic fatty acids. The fourth short-chain fatty acid in question showed an ECL value of 17.06 (methyl ester) with a

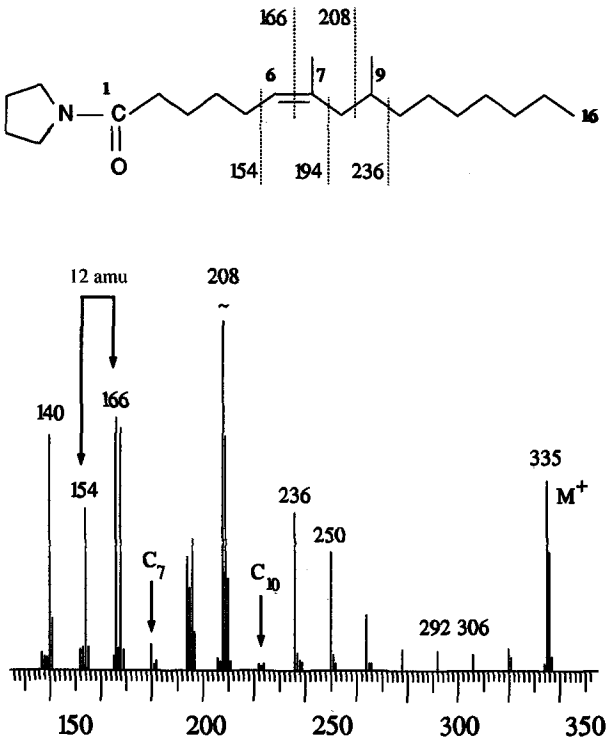


FIG. 3. Partial mass spectrum of 7,9-dimethyl-6-hexadecenoic acid pyrrolidide **9** (×6 expanded).

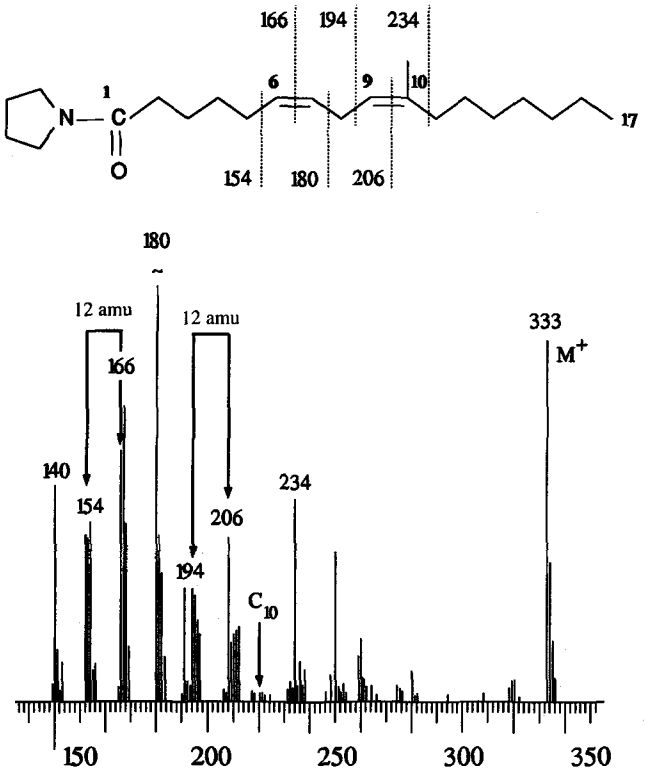


FIG. 4. Partial mass spectrum of 10-methyl-6,9-heptadecadienoic acid pyrrolidide **11** (×20 expanded).

molecular ion peak at *m/z* 294 compatible with a methyl branched 18:2 acid structure. Upon catalytic hydrogenation, it was converted into a branched saturated methyl ester (M^+ 298). The mass spectrum of pyrrolidide **11** (Fig. 4) showed a molecular ion peak at *m/z* 333, confirming the 18:2 acid structure.

Differences of 12 amu between fragmentation ions at *m/z* 154 and 166 and *m/z* 194 and 206 indicated Δ6 and Δ9 unsaturations, respectively, and the presence of a methyl branch at C-10 was indicated by a low peak at *m/z* 220 (C_{10}), flanked by two elevated peaks at *m/z* 206 (C_9) and 234 (C_{11}). Thus, another new fatty acid, namely 10-methyl-6,9-17:2, was identified. Furthermore, we readily identified the straight-chain 6,9-heptadecadienoic acid because its pyrrolidide displayed a molecular ion peak at *m/z* 319 and key fragmentation ions at *m/z* 154 and 166 and *m/z* 194 and 206.

PUFAs. Gas chromatograms of FAME showed several unusual peaks, especially in lipids from colder water specimens, showing relatively high molecular ions. For example, a major component had an ECL value of 25.40 on Carbowax-20M and 22.90 on OV-1 (22.95 on DB-1). Upon catalytic hydrogenation, it was converted to the lignoceric acid methyl ester (24:0), which indicated that there was no branching. The MS of the methyl ester showed a molecular ion peak at *m/z* 372, suggesting a 24:5 fatty acid structure. This 24:5 structure was confirmed by MS of the pyrrolidide derivative as previously described (7). Its MS showed a molecular ion peak at *m/z* 411 (24:5) and differences of 12 amu between the following homologous fragments: *m/z* 154 and 166 (Δ6), *m/z* 194 and 206 (Δ9), *m/z* 234 and 246 (Δ12), *m/z* 274 and 286 (Δ15), *m/z* 314 and 326 (Δ18). These key fragments indicated a methylene-interrupted polyenoic fatty acid, namely 6,9,12,15,18-24:5. Similarly, 9,12,15,18,21-24:5 and 6,9,12,15,18,21-24:6 were detected in all samples.

In FTIR spectra, all unsaturated FAMEs exhibited absorptions near 3010 cm^{-1} and 700 cm^{-1}, but no absorption near 960 cm^{-1}, that characterized only a (*Z*)-configuration (21).

Fatty aldehydes, long-chain dimethyl acetals. During GC analyses of the crude FAME mixtures, we observed several unexpected peaks in all samples, in ratios up to 10% depending on the samples studied, relative to the crude total mixture arising from the transmethylation reaction of the total lipids. GC/MS studies showed that these peaks corresponded to fatty aldehydes and dimethyl acetals with even and odd carbon numbers ranging from 14 to 20. The major components were thus octadecanal and its dimethyl acetal. As examples, all the MS of dimethyl acetals displayed the fragmentation ion at *m/z* 75 as the base peak. Other significant ions corresponded to the $(M - 31)^+$ ions, i.e., *m/z* 283 for octadecanal dimethyl acetal. Such fatty aldehydes indicated the presence of plasmalogens (1-*O*-alkyl-1-enyl-2-acyl lipids).

DISCUSSION

The fatty acid composition of the gorgonian specimens collected during the same period was generally quite similar

(Table 1). The saturated fatty acid content of warmer water specimens was about twice that of colder water specimens. Fatty acids that are usually bacterially derived, such as *iso* and *anteiso* branched acids, were only present at low percentages, despite the fact that gorgonians are filter-feeding organisms.

An interesting result of our study was the identification of four closely related branched, unsaturated fatty acids, three of which proved to be new compounds. All of these acids contained Δ6 unsaturations, also encountered in some straight-chain monoenoic acids (Table 1), mainly 6-hexadecenoic acid. The rare 7-methyl-6-hexadecenoic acid occurring in easily-measured proportions of the total fatty acid mixtures was previously found in sponges (19,20) and in whales (22). Furthermore, an isomer of this compound was the newly described 10-methyl-6-hexadecenoic acid that eluted just prior to 7-methyl-6-hexadecenoic acid on GC. Two theories have been proposed regarding the biosynthetic origin of such branched, unsaturated fatty acids. First, it was proposed that the introduction of the double bond in 7-methyl-8-hexadecenoic acid could occur after chain-elongation and introduction of the methyl branch (23). Another theory implies that the insertion of a methylene group across a preexisting ethylenic bond and a subsequent rearrangement probably involved bacterial systems (16). This second theory is supported by the fact that 7-methyl-7-hexadecenoic and 7,8-dimethyl-7-hexadecenoic acids are found together at high levels in certain fish oils (16). In this respect, the 7-methyl-7-hexadecenoic acid occurring in the jellyfish *Physalia physalis* is thought to originate from 7-hexadecenoic acid (24), which is also present in this organism. Such branched, monoenoic fatty acids may be biosynthetic intermediates to cyclopropane fatty acids from the corresponding monoenoic acid, with *S*-adenosylmethionine donating the methyl branch (24). In our gorgonians, the co-occurrence of the isomeric 7-methyl-6-hexadecenoic and 10-methyl-6-hexadecenoic acids, together with the straight-chain 6-hexadecenoic acid, also seems to corroborate the second theory as the latter acid is a precursor. If so, 10-methyl-6-hexadecenoic acid should arise from a dienoic fatty acid such as 6,9-hexadecadienoic acid, even though this acid, was not detected in the fatty acid mixtures. This possibility would involve a subsequent reduction step. Nevertheless, it cannot be excluded that 10-methyl-6-hexadecenoic acid could arise from 10-methyl-hexadecanoic acid, a relatively usual fatty acid of bacterial origin (17,25), because gorgonians possess ample Δ6 desaturase activity. However, no traces of corresponding 7-methyl- and 10-methylhexadecanoic acids were detected in the gorgonians.

In the same way, hypothetical pathways may also be suggested for the other related new fatty acids, namely 7,9-dimethyl-6-hexadecenoic and 10-methyl-6,9-heptadecadienoic. These compounds also possess Δ6 unsaturation and a methyl branch at either the C-7 or C-10 position as common features. As suggested above, a second unsaturation (Δ9) could exist. Thus, a methylene group could be inserted to yield, for example, 7,9-dimethyl-6-hexadecenoic acid after rearrangement to a 9-methyl branch and reduction, despite the absence of 6,9-hexadecadienoic acid in the mixture. The fact that we identified 7,9-dimethyl-6-heptadecenoic acid instead of the expected 7,10-dimethyl-6-hexadecenoic acid emphasizes the interest of determining the mode of biosynthesis of these new branched fatty acids. In contrast, 6,9-heptadecadienoic acid was present and perhaps a precursor in the biosynthesis of 10-methyl-6,9-heptadecadienoic acid, implying a unique 10-methyl branching. According to these speculations, the most plausible biosynthetic pathway would involve methylene insertion across double bonds. However, further biosynthetic experiments need to be performed to establish this with certainty.

Even taking into consideration possible differences due to the different extraction methods used, it is noteworthy that a low percentage of lipids, but a high level of PUFA, was observed during the cool season, whereas a high percentage of lipids, but a low level of PUFA, was observed for gorgonians collected in warmer waters. Such a low percentage of lipids was also reported for a Caribbean gorgonian harvested in December and extracted by the Bligh and Dyer method (6). Increased lipid content is often reflected by increases in TGs and wax esters. Thus, a high level of wax esters could account for the decrease of PUFA content (5,6). Furthermore, the possibility of lipid autoxidation may not be excluded, which would imply loss of PUFA (6). The influence of environmental temperature seems the most plausible explanation. Temperature is known to be one of the major environmental changes to which marine organisms are subjected. It is generally reported that increases in the highly unsaturated fatty acids of marine organisms occur in colder waters (26,27). This is assumed to be a way of adapting to the environment, in that temperature-dependent changes in lipids influence the thermal flexibility of cell membranes, ensuring correct physicochemical properties (28). As an exception, arachidonic acid was present at high levels in all the specimens studied here. This was probably correlated with the fact that arachidonic acid is known primarily as a component of polar lipids.

Dietary input or a symbiotic origin may also account for the variations in PUFA levels. Cnidarians are hosts for photosynthetic Zooxanthellae, especially in shallow waters (5). Dinoflagellate endosymbionts are widely distributed among marine invertebrates, and their lipids are characterized by high PUFA concentrations (29). In a previous study, we reported the fatty acid compositions of five *Palythoa* (Zoantharia) from the Senegalese coast and their associated organisms (10). Four of them harvested in shallow waters were associated with Zooxanthellae, whereas *Palythoa senegambiensis*, which lives at depths of 12 to 20 meters, was devoid of endosymbiotic Zooxanthellae. In fact, at 30 meters depth on average, light is insufficient to sustain photosynthetic processes because of the constant turbidity of the ocean. This observation was also documented in our recent work showing that 23,24-dimethylcholesta-5,22-dien-3β-ol, a gorgosterol precursor specific to Zooxanthellae, was present only in shallow

water *Cinachyrella* sponges, whereas the corresponding deep-water specimens did not possess this sterol (30). Thus, PUFA would not seem to originate from symbiotic Zooxanthellae.

An interesting result was the identification at high levels of the 6,9,12,15,18,-24:5 and quite rare 6,9,12,15,18,21-24:6 fatty acids in gorgonian specimens from colder waters. These fatty acids have only been reported at similar levels in two gorgonians (7) and in some other Cnidarians, such as Pennatularia and Alcyonaria (7). The 6,9,12,15,18,21-24:6 fatty acid was previously found in Crinoidea and Ophiuroidea (31). These tetracosapolyenoic fatty acids probably arise from arachidonic and icosapentaenoic acids, although further experiments are required to clarify this point. According to preliminary investigations (Mirallès, J., and Galonnier, R., unpublished results), high proportions of tetracosapolyenoic acids would seem characteristic of the total lipids of gorgonians collected in colder waters. To further elucidate this point, studies are currently underway in our laboratory to investigate a wide range of available gorgonians.

ACKNOWLEDGMENTS

We wish to thank Aimée Kornprobst for technical assistance, Gilbert Nourrisson (Laboratoire de Synthèse Organique, CNRS, Faculté des Sciences, Nantes) for GC/MS experiments, and Dr. Pierre Doumenq (Centre de Spectroscopie Moléculaire, Faculté des Sciences, Marseille) for GC/FTIR experiments.

REFERENCES

1. Mirallès, J., Galonnier, R., Njinkoué, J-M., Samb, A., Gaydou, E.M., and Kornprobst, J.M. (1992) *Comp. Biochem. Physiol. 103B*, 1039–1041.
2. Roussis, V., Fenical, W., Mirallès, J., and Kornprobst, J.M. (1991) *New J. Chem. 15*, 959–961.
3. Joseph, J.D. (1979) *Progr. Lipid Res. 18*, 1–30.
4. Isay, S.V., and Busarova, N.G. (1984) *Comp. Biochem. Physiol. 77B*, 803–810.
5. Joseph, J.D. (1989) in *Marine Biogenic Lipids, Fats and Oils* (Ackman, R.G., ed.) pp. 51–143, CRC Press, Boca Raton.
6. Hooper, S.N., and Ackman, R.G. (1972) *Lipids 7*, 624–626.
7. Vysotskii, M.V., and Svetashev, V.I. (1991) *Biochim. Biophys. Acta 1083*, 161–165.
8. Light, R.J. (1973) *Biochim. Biophys. Acta 296*, 461–466.
9. Weinheimer, A.J., and Spraggins, R.L. (1969) *Tetrahedron Lett. 5185*:5188.
10. Mirallès, J., Diop, M., Ferrer, A., and Kornprobst, J.M. (1989) *Comp. Biochem. Physiol. 94B*, 91–97.
11. Grasshoff, M. (1988) *Atlantide Report 14*, 91–147.
12. Sourie, R. (1954) *Mémoires Inst. Fr. Afr. Noire, Dakar 38*, 70.
13. Leung Tack, D. (1983) in *Chimie Marine pour le Développement* (Kornprobst, J.M., ed.) *91–92*, pp. 118–123, ENDA TM, Dakar.
14. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
15. Metcalfe, L.D., and Schmitz, A.A. (1961) *Anal. Chem. 33*, 363–364.
16. Ratnayake, W.M.N., Olson, B., and Ackman, R.G. (1989) *Lipids 24*, 630–637.
17. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi C. (1981) *Lipids 16*, 631–646.
18. Barnathan, G., Mirallès J., Gaydou, E.M., Boury-Esnault, N., and Kornprobst, J.M. (1992) *Lipids 27*, 779–784.
19. Carballeira, N.M., and Restituyo, J. (1991) *J. Nat. Prod. 54*, 315–317.
20. Barnathan, G., Kornprobst, J.M., Doumenq, P., Mirallès, J., and Boury-Esnault, N. (1993) *J. Nat. Prod. 56*, 2104–2113.
21. Doumenq, P., Guiliano, M., Bertrand, J.C., and Mille, G. (1990) *Appl. Spectrosc. 44*, 1355–1359.
22. Pascal, J.C., and Ackman, R.G. (1975) *Lipids 10*, 478–482.
23. Carballeira, N.M., and Maldonado, M.E. (1988) *Lipids 23*, 690–693.
24. Stillway, L.W. (1976) *Comp. Biochem. Physiol. 53*, 535–537.
25. Campbell, I.M., and Naworal, J. (1969) *J. Lipid Res. 10*, 593–598.
26. Beninger, P.G., and Stephan, G. (1985) *Comp. Biochem. Physiol. 81*, 591–601.
27. Reinhardt, S.B., and Van Vleet, E.S. (1986) *Mar. Biol. 91*, 149–159.
28. Neidleman, S.L. (1987) *Biotechnol. Gen. Eng. Rev. 5*, 245–268.
29. Bishop, D.G., and Kenrick, J.R. (1980) *Lipids 15*, 799–804.
30. Barnathan, G., Mirallès, J., Gaydou, E.M., Boury-Esnault, N., and Kornprobst, J.M. (1992) *Lipids 27*, 779–784.
31. Tagaki, T., Kaneniwa, M., and Itabashi, Y. (1986) *Lipids 21*, 430–433.

[Received September 27, 1993, and in final revised form March 14, 1995; Revision accepted March 20, 1995]

The Rare Caribbean Sponge *Leucosolenia canariensis:* Phospholipid Fatty Acids and Sterols

Néstor M. Carballeira* and Fathi Shalabi

Department of Chemistry, University of Puerto Rico, San Juan, Puerto Rico 00931-3346

ABSTRACT: The phospholipid fatty acid composition of the Calcarean sponge *Leucosolenia canariensis* was studied, and no $\Delta^{5,9}$ fatty acids were detected. These results are in contrast to the phospholipids from sponges belonging to the class Demospongiae where Δ5,9 fatty acids are predominant. Odd branched-chain fatty acids between 17 and 19 carbons accounted for 26% of the total fatty acids of *L. canariensis*, while straight-chain fatty acids between 16 and 22 carbons accounted for 61% of the total fatty acid composition. The sterol composition of *L. canariensis* is also reported, and only $\Delta^{5,7,22}$ sterols were observed.

Lipids 30, 467–470 (1995).

Fatty acids and sterols from marine sponges are metabolites of particular interest for the unusual array of new structures that have been isolated (1). In particular, the class Demospongiae has been recognized as having very long-chain fatty acids (typically between 26 and 30 carbons) with a characteristic $\Delta^{5,9}$ diunsaturation pattern (2). However, little is known about the specific chemical structures of fatty acids of lipids of the Calcarea. In the only study of Calcarea, Bergquist *et al.* (3) analyzed the lipids of the genus *Clathrina* and *Leucettusa* (subclass Calcinea), and *Sycon* (subclass Calcarea). This study revealed that the only difference in fatty acid profiles between the Demospongiae and Calcarea were the smaller percentages of C_{24}–C_{30} acids and fewer unsaturated fatty acids in the class Calcarea. The species *Leucettusa lancifer* was atypical within the Calcarea in that the fatty acid chain lengths were no greater than 22 carbons (3). However, no specific chemical structures for these fatty acids were reported. Moreover, the order Leucosolenida has not been studied for fatty acid composition.

The sterol composition of the Calcarea has also been studied, indicating that they do contain small amounts of sterols, the Δ^5 sterols between C_{27}–C_{29} carbons being predominant in these sponges (4). The sterol composition of the Calcarea compares favorably to the class Demospongiae (5). However, there are no reports of the sterol composition of the order Leucosolenida within the Calcarea. One reason might be that the Leucosolenid sponges are difficult to find and/or collect.

In our ongoing research of the fatty acid composition of Caribbean sponges, we were fortunate to find the rare Calcarean sponge *Leucosolenia canariensis* (genus Leucosolenia, order Leucosolenida, subclass Calcaronea). The phospholipid fatty acid distribution and the sterol composition of *L. canariensis* have not been studied before and are, therefore, presented herein.

MATERIALS AND METHODS

Instrumentation. Gas chromatography (GC) spectra were recorded on a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) equipped with a flame-ionization detector and a DB-1 (J&W Scientific, Folsom, CA) nonpolar fused silica capillary column (30 m × 0.25 mm i.d.) containing dimethylpolysiloxane (carrier gas He). Electron ionization/mass spectrometry (MS) data was collected in a 5972A MS ChemStation operated at 70 eV (Hewlett-Packard) and equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) crosslinked with 5%-phenyl methylpolysiloxane. The temperature program was as follows: 130°C for 2 min, then increased at 3°C/min to 270°C and maintained for 40 min. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a General Electric GN-300 (300 MHz) (Broker Instruments, Billerica, MA) spectrometer in $CDCl_3$. Preparative high-performance liquid chromatography (HPLC) was carried out on a Waters Associates HPLC system (M 510 pump, Waters 410 differential refractometer; Milford, MA), using an Altex Ultrasphere ODS-2 column (25 cm × 10 mm i.d.; Berkeley, CA) and methanol as solvent.

Sampling and lipid analyses. Leucosolenia canariensis was collected north of Puerto Rico, at a depth of 10 m. The sponge (*ca.* 100 g) was washed in seawater, carefully cleaned of all nonsponge debris, and cut into small pieces. Immediate extraction after collection with 200 mL of chloroform/methanol (1:1, vol/vol) yielded the total lipids (100 mg). The neutral lipids, glycolipids, and phospholipids (60 mg) were separated

*To whom correspondence should be addressed at Dept. of Chemistry, University of Puerto Rico, P.O. Box 23346, San Juan, Puerto Rico 00931-3346.

Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

by column chromatography on silica gel (60–200 mesh) using the procedure of Privett *et al.* (6). The phospholipid classes were fractionated by preparative thin-layer chromatography (TLC) using Silica gel 60 and $CHCl_3/CH_3OH/NH_4OH$ (65:35:5, by vol) as solvent, affording mainly phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. The fatty acyl components of these phospholipids were obtained as their methyl esters by reaction with methanolic hydrogen chloride (7) followed by silica gel chromatography with hexane/diethyl ether (9:1, vol/vol) as eluent.

Hydrogenations were carried out in 10 mL of absolute methanol and catalytic amounts of platinum oxide (PtO_2). The double-bond positions of the mono- and dienoic fatty acids were determined by preparing the corresponding dimethyl disulfide derivatives by dissolving the esters or dimethyl acetals (2 mg) in dimethyldisulfide (0.2 mL) and adding a solution (0.05 mL) of I_2 in Et_2O (60 mg/mL), heating the solution at 50°C for 24 h, followed by the standard work-up (8). Spectral data for the dimethyldisulfide adducts of the unsaturated aldehydes were as follows: 10,11-*bis*(methylthio)nonadecanal. MS *m/z* (relative intensity) 374 (M^+, 3), 201($C_{11}H_{21}SO^+$, 27),173 ($C_{10}H_{21}S^+$, 100), 157 (5), 135 (9), 124 (4), 121 (2), 109 (8), 107 (7), 97 (10), 95 (26), 93 (12), 87 (24), 83 (40), 81 (31), 79 (15), 74 (13). 10,11-*bis*(methylthio)Heneicosanal. MS *m/z* (relative intensity) 402 (M^+, 2), 202 (13), 201($C_{11}H_{21}SO^+$ and $C_{12}H_{25}S^+$, 88), 200 (10), 174 (4), 157 (3), 143 (3), 135 (10), 123 (4), 113(7), 111 (8), 109 (10), 107 (9), 101 (5), 97 (30), 95 (33), 93 (16), 91 (8), 87 (31), 85 (11),83 (43), 81 (39), 79 (20), 74 (16), 71 (18).

For the isolation of the sterol mixture, the previously isolated neutral lipids were chromatographed on a silica gel column [ether/hexane (1:1, vol/vol) as eluant] to provide the sterol mixture. The sterol mixture was recrystallized from both CH_3OH and CH_3CN (Carballeira, N.M., unpublished data). The individual sterols were fractionated by HPLC on an Altex ODS-2 column and then run on GC to confirm purity.

RESULTS AND DISCUSSION

The phospholipid fatty acid composition of *L. canariensis* is presented in Table 1. In contrast to the class Demospongiae, no Δ5,9 fatty acids were observed in this Calcarean sponge. The longest fatty acids in the phospholipids of the sponge were the polyunsaturated fatty acids 4,7,10,13,16,19-docosahexaenoic acid (22:6) and 7,10,13,16-docosatetraenoic acid (22:4).The principal fatty acids in *L. canariensis* were hexadecanoic acid (16:0) and octadecanoic acid (18:0), which together accounted for 23% of the total fatty acid composition of this sponge. Branched-chain fatty acids were the predominant classes in *L. canariensis,* with odd-chain fatty acids accounting for 26% of the total fatty acid composition and even-chain branched fatty acids for 12%. In this regard, the *iso/anteiso* pair of nonadecanoic acids were in considerable abundance. *Leucosolenia canariensis* also presented the related n-7 monounsaturated fatty acids 9-hexadecenoic acid and 11-octadecenoic acid (Table 1). The double-bond positions were unambiguously determined from the mass spectra of the corresponding dimethyldisulfide adducts (8).

TABLE 1
Fatty Acids and Aldehydes from *Leucosolenia canariensis*

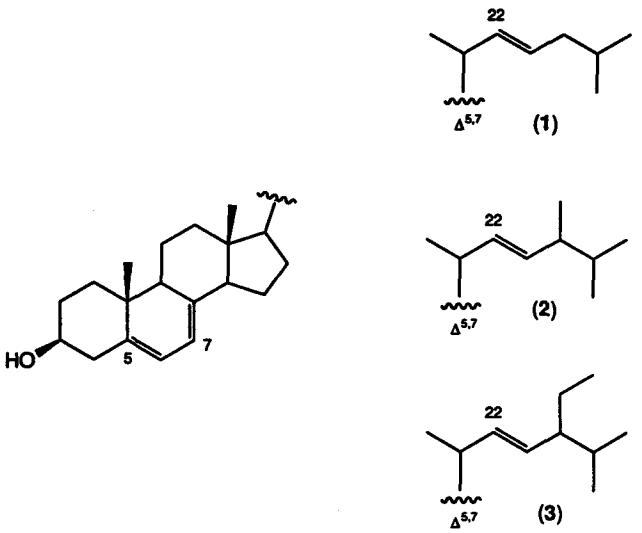
Compounds	Abundance (wt%)
Fatty acids	
Tetradecanoic (14:0)	7.6
4,8,12-Trimethyltridecanoic (16:0)	3.5
13-Methyltetradecanoic (15:0)	3.5
Pentadecanoic (15:0)	1.7
9-Hexadecenoic (16:1)	0.7
11-Hexadecenoic (16:1)	0.2
Hexadecanoic (16:0)	13.5
15-Methylhexadecanoic (*i*-17:0)	4.7
14-Methylhexadecanoic (*ai*-17:0)	1.4
Heptadecanoic (17:0)	2.0
16-Methylheptadecanoic (*i*-18:0)	8.4
9-Octadecenoic (18:1)	0.1
11-Octadecenoic (18:1)	6.1
Octadecanoic (18:0)	9.8
17-Methyloctadecanoic (*i*-19:0)	6.1
16-Methyloctadecanoic (*ai*-19:0)	9.8
Nonadecanoic (19:0)	9.6
13-Eicosenoic (20:1)	0.5
15-Eicosenoic (20:1)	0.1
Eicosanoic (20:0)	1.9
4,7,10,13,16,19-Docosahexaenoic (22:6)	1.0
7,10,13,16-Docosatetraenoic (22:4)	5.9
Aldehydes	
Heptadecanal (17:0)	1.2
Octadecanal (18:0)	0.1
10-Nonadecenal (19:1)	0.5
10-Heneicosenal (21:1)	0.1

Dimethyl acetals of aldehydes were also present in the methyl ester mixture from the sponge. The dimethylacetal derivatives of the saturated aldehydes heptadecanal and octadecanal exhibited a base peak at *m/z* 75 and $[M - 31]^+$ ions at *m/z* 269 and at *m/z* 283, respectively. The previously unrecognized 10-nonadecenal (19:1) and 10-heneicosenal (21:1), which predominated in phosphatidylethanolamine, were also identified in *L. canariensis*. Their dimethyl acetals probably accompanied the methyl esters as "artifacts" in the transesterification. The dimethyl acetal derivatives of both aldehydes exhibited a base peak at *m/z* 75 and $[M - 31]^+$ ions at *m/z* 295 and at *m/z* 323, respectively. The double-bond position in the chains was determined by dimethyldisulfide derivatization of small amounts of free aldehydes accompanying the methyl esters and dimethyl acetals. For example, 10-nonadecenal afforded, upon reaction with dimethyldisulfide and iodine in diethyl ether, the 10,11-*bis*(methylthio)nonadecanal. The electron impact mass spectrum of the latter derivative displayed predominant fragments at *m/z* 201 $[C_{11}H_{21}SO]^+$ and at *m/z* 173 $[C_{10}H_{21}S]^+$, resulting from preferential cleavage between C-10 and C-11, and this indicates a double bond at C-10. The 10-heneicosenal (21:1) was characterized in a similar way.

The 10,11-*bis*(methylthio)heneicosanal provided major fragments with the same mass at *m/z* 201$[C_{11}H_{21}SO]^+$ and at *m/z* 201 $[C_{12}H_{25}S]^+$, which also indicates a double bond at C-10. However, in this particular case, a single ion peak was observed for both fragments at *m/z* 201.

The sterol composition of *L. canariensis* was also carefully studied and is presented in Table 2. The ^{1}H NMR spectrum of the sterol mixture presented signals characteristic of a $\Delta^{5,7}$ nucleus (9). For example, the C18 and C19 methyl groups were observed at 0.63 and 0.94 ppm, respectively, as well as the two characteristic ring-olefinic signals deshielded to δ 5.38 and 5.58 ppm, corresponding to the $\Delta^{5,7}$ protons.

The HPLC chromatogram of the sterol mixture presented only three main peaks (Scheme 1). The first peak (27% of the total sterol composition) was characterized as cholest-5,7,22-trien-3ß-ol (**1**).The mass spectrum of **1** displayed a $[M]^+$ at 382 and fragments at *m/z* 271 $[C_{19}H_{27}O]^+$ (loss of side chain) and at *m/z* 253 $[C_{19}H_{25}]^+$ (loss of side chain and water) corresponding to an unsaturated side chain characteristic of sterols with the $\Delta^{5,7}$ nucleus (9). The double bond at C22 was confirmed from the existence of an ion at *m/z* 323 (loss of the terminal isopropyl group and water). The second HPLC peak (34% of the total mixture) was identified as 24-methylcholest-5,7,22-trien-3ß-ol (**2**). Diagnostic fragments for **2** also occurred at *m/z* 396 $[M]^+$, *m/z* 378 (loss of water), *m/z* 363 (loss of methyl and water), *m/z* 337 (loss of the terminal isopropyl group and water), and at *m/z* 253 (loss of side chain and water). The C28 methyl signal appeared as a doublet at δ 0.91 ppm. Finally, the third HPLC peak (38% of the total mixture) contained a sterol with $[M]^+$ at 410. This was shown to be 24-ethylcholest-5,7,22-trien-3ß-ol (**3**) by comparison of its mass spectrum with the literature (9). The mass spectrum of **3** presented fragments at *m/z* 392 (loss of water), *m/z* 377 (loss of methyl and water), *m/z* 351(loss of the terminal isopropyl group and water), and at *m/z* 253 (loss of side chain and water).The C29 methyl group appeared on ^{1}H NMR as a triplet at 0.84 ppm, confirming the presence of a methyl group at C-24 (9).



SCHEME 1

TABLE 2
Sterols from *Leucosolenia canariensis*

Sterols	Abundance (wt%)
Cholest-5,7,22-trien-3ß-ol (**1**)	28
24-Methyl-5,7,22-trien-3ß-ol (**2**)	34
24-Ethylcholest-5,7,22-trien-3ß-ol (**3**)	38

Our study of the phospholipid fatty acid composition of *L. canariensis* is important for several reasons. First, we have shown that *L. canariensis* does not have any Δ5,9 fatty acids. This is an interesting observation that could have taxonomic relevance when we compare this Calcarean sponge to other sponges from the class Demospongiae, a class where most of the sponges do have Δ5,9 fatty acids in their phospholipids. A second interesting observation can be made as to the relative fatty acid chain lengths of sponges within the Calcarea. A comparison of the fatty acid profile of *L. canariensis* with that reported for other Calcarean sponges reveals that it compares favorably to that of the Leucettida sponge *Leucettusa lancifer*, a sponge previously analyzed by Bergquist *et al.* (3). Both sponges had fatty acids with chain lengths up to only 22 carbons. Other Calcarean sponges from the order Clathrinida and Sycettida do show predominantly fatty acids with very long chain lengths up to 26 carbons. Although this comparison of the two sponges is not strictly correct, i.e., they were analyzed under different conditions, our results seem to imply that the order Leucosolenida is closer to the order Leucettida, but farther away from the orders Clathrinida and Sycettida.

The sterol composition of *L. canariensis* also supports the different chemical composition of the Leucosolenida as compared to the Clathrinida and Sycettida (4). *Leucosolenia canariensis* had only $\Delta^{5,7,22}$ sterols, while the other Calcarean sponges have been reported to have mainly Δ^5 sterols (4). Unfortunately, we could not find any reports on the sterol composition of *Leucettusa lancifer*.

The last interesting observation in *L. canariensis* is the report of the aldehydes 10-nonadecenal and 10-heneicosenal, probable "artifacts" of transesterification. Work is in progress in our laboratory elucidating the origin of these unusual lipids in marine sponges.

ACKNOWLEDGMENTS

We thank Dr. Vance Vicente for the collection and classification of the sponge. We thank the Puerto Rico (EPSCoR) Tropical Marine Biotechnology Center for financial support as well as the National Science Foundation MRCE program. This work was also supported by the National Institutes of Health (NIH-MBRS Program) under Grant No. S06 GM08102-20.

REFERENCES

1. Djerassi, C., and Lam, W.-K. (1991) *Acc. Chem. Res. 24*, 69–75.
2. Carballeira, N., and Shalabi, F. (1994) *J. Nat. Prod. 57*, 1152–1159.

3. Bergquist, P., Lawson, M., Lavis, A., and Cambie, R. (1984) *Biochem. Syst. Ecol. 12*, 63–84.
4. Bergquist, P., Lavis, A., and Cambie, R. (1986) *Biochem. Syst. Ecol. 14*, 105–112.
5. Bergquist, P., Karuso, P., Cambie, R., and Smith, D. (1991) *Biochem. Syst. Ecol. 19*, 17–24.
6. Privett, O.S., Dougherty, K.A., Erdahl, W.L., and Stolyhwo, A. (1973) *J. Am. Oil Chem. Soc. 50*, 516–520.
7. Carreau, J.P., and Dubacq, J.P. (1978) *J. Chromatogr. 151*, 384–390.
8. Francis, G., and Veland, K. (1981) *J. Chromatogr. 219*, 379–384.
9. Delseth, C., Tolela, L., Scheuer, P., Wells, R., and Djerassi, C. (1979) *Helv. Chim. Acta 62*, 101–109.

[Received December 5, 1994, and in revised form March 7, 1995; Revision accepted March 15, 1995]

Dietary Manipulation of Long-Chain Polyunsaturated Fatty Acids in the Retina and Brain of Guinea Pigs

Harrison S. Weisinger[a], Algis J. Vingrys[a] and Andrew J. Sinclair[b,*]

[a]Department of Optometry, University of Melbourne, Parkville, 3052, Victoria, Australia and
[b]Department of Food Science, Royal Melbourne Institute of Technology, Melbourne, 3000, Victoria, Australia

ABSTRACT: High levels of n-6 docosapentaenoic acid (22:5n-6) have been reported in the retina of guinea pigs fed commercially-prepared grain-based rations (commercial diet). In rats and monkeys, high levels of 22:5n-6 are an indicator of n-3 polyunsaturated fatty acid (PUFA) deficiency. We have examined the fatty acid composition of the retina and brain in guinea pigs fed a commercial diet or one of three semi-purified diets containing three different levels of n-3 PUFA. The diets comprised a diet deficient in n-3 PUFA (semi-purified diet containing safflower oil), two diets containing α-linolenic acid (standard commercial laboratory diet and semi-purified diet containing canola oil), and a diet containing α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid (DHA) (semi-purified diet containing canola oil, safflower oil, and fish oil). Two groups of guinea pigs were given the diets from day 1 to 4 wk or day 1 to 8 wk, when they were sacrificed and the retinal tissues were extracted and analyzed for PUFA content by gas–liquid chromatography. Fatty acid analyses of the retinal phospholipids of the four-week-old animals revealed that the group fed DHA (from the fish oil) had the highest level of DHA (32%), compared with values of 19 and 13% for the groups fed canola oil diet and commercial diet, respectively, and 2% for the group fed the diet deficient in n-3 PUFA. The levels of 22:5n-6 in the retinal lipids were inversely related to the DHA values, being 0.6, 6.6, 11.4, and 20.6 for the fish oil, canola oil, commercial diet, and safflower oil diet groups, respectively. The long-chain PUFA profiles in the brain phospholipids of the four-week-old group were similar to those from the retina. The retinal PUFA values for the eight-week-old animals were similar to the four-week-old group. The safflower oil diet induced a greater deficit of DHA in retinal lipids than has been reported in rats and monkeys fed similar diets. The guinea pigs fed the commercial diet had retinal and brain PUFA patterns similar to that produced by n-3 PUFA-deficient diets in rats and monkeys. Guinea pigs fed the canola oil diet had significantly greater retinal DHA levels than those fed the commercial diet, but lower than those fed fish oil. The data suggest that the guinea pig has a reduced capacity for DHA synthesis from α-linolenic acid as compared with other mammals. Supplementation of guinea pig diets with fish oil produced high retinal and brain DHA levels and prevented the accumulation of 22:5n-6.
Lipids 30, 471–473 (1995).

Docosahexaenoic acid (DHA) (22:6n-3) is the major polyunsaturated fatty acid (PUFA) in brain grey matter and retina of most mammalian species (1–3). Animals fed n-3 PUFA-deficient diets develop low levels of DHA in the brain and retina and an increase in n-6 docosapentaenoic acid (22:5n-6) (3–6). High levels of 22:5n-6 and low levels of 22:6n-3 in tissue phospholipids are considered to be a biochemical marker of n-3 PUFA deficiency (3–6). Such fatty acid changes are associated with abnormal electroretinograms in rats and monkeys (3–6), and alterations in enzyme activities in neural tissue (4). In 1986, Leat *et al.* (7) reported that commercial diet-fed guinea pigs contained a high level of 22:5n-6 and a relatively low level of DHA in the retinal lipids, suggesting that the commercial diet used was deficient in α-linolenic acid. The guinea pig commercial diet contained linoleic and α-linolenic acid as the only dietary PUFA. The finding that the control guinea pigs showed biochemical evidence of reduced DHA levels might explain why Leat *et al.* (7) were not able to detect electroretinogram differences between the control guinea pigs and those fed a semi-purified diet containing linoleic acid as the only PUFA (n-3-deficient diet).

The purpose of this study was to establish whether guinea pigs fed semi-purified diets containing α-linolenic acid and/or DHA could prevent the accumulation of docosapentaenoic acid (22:5n-6) in retinal and brain tissue.

MATERIALS AND METHODS

Animals and diets. Sixteen commercial diet-fed female guinea pigs (in groups of four) were weaned onto a guinea pig commercial diet (Barrastock Guinea Pig Food; Barrastock, Melbourne, Australia), or one of three semi-purified diets

*To whom correspondence should be addressed at the Department of Food Science, Royal Melbourne Institute of Technology, GPO Box 2476V, Melbourne, 3001, Victoria, Australia.

Abbreviations: DHA, docosahexaenoic acid (22:6n-3); PUFA, polyunsaturated fatty acids.

Copyright © 1995 by AOCS Press

based on those described by Leat *et al.* (7). The diets contained (g/kg) casein 300, sucrose 100, glucose 70, starch 200, solkafloc 100, oil 100, kaolin 30, L-arginine 3, DL-methionine 2, mineral mix 68, and vitamin pre-mix 27. All diets were supplemented with 10 g fresh carrots daily, and water containing ascorbic acid (400 mg/L) was freely available. Details of the vitamin premix and mineral mix are given in Leat *et al.* (7). The guinea pig commercial diet contained 14 g/kg linoleic acid, 1.6 g/kg α-linolenic acid, and no long-chain PUFA. The semi-purified diets were identical, except for the added oils. The n-3-deficient diet contained safflower oil as the sole source of fat (71 g/kg linoleic acid, <0.7 g/kg α-linolenic acid), the second diet contained canola oil which provided linoleic acid and α-linolenic acid as the only PUFA (20 g/kg linoleic acid, 8 g/kg α-linolenic acid), and the third diet contained a mixture of safflower oil, canola oil, and fish oil, thus providing eicosapentaenoic acid (4.5 g/kg) and DHA (2.6 g/kg) as well as linoleic acid (30.5 g/kg) and α-linolenic acid (3.8 g/kg). The fish oil was supplied in gelatin-coated powder form (Dry n-3®; DanoChemo, Ballerup, Norway). Guinea pig dams were reared on these diets to four months of age, at which time they were joined with commercial diet-fed males. Six offspring from each diet group were sacrificed at 28 ± 2 d, and a further 12 animals from each of the four diets were sacrificed at 56 ± 4 d. The young guinea pigs consumed solid food from about seven days of age. There were an equal number of animals of each sex in each diet group. The animals were sacrificed following intraperitoneal injection of sodium pentabarbitone (100 mg/kg body weight) (Boehringer Ingelheim, Artarmon, NSW, Australia). The retinas were removed within 2 min, washed in ice-cold phosphate-buffered saline, blotted dry, and immediately placed in chloroform/methanol (2:1, vol/vol) in glass Teflon-lined screw-capped tubes. The brains were also removed (from the young group) within 2 min, halved down the mid-line, diced, and also immediately placed into chloroform/methanol solution.

Lipid analyses. Lipids were extracted from the retinas and brain tissue using chloroform/methanol, and the phospholipids were separated from the neutral lipids by thin layer-chromatography (8). The methyl esters of the phospholipid fatty acids were formed by a saponification step using KOH followed by transesterification in BF_3 in methanol (8), and the fatty acid methyl esters were separated by capillary gas–liquid chromatography using a 50 m × 0.32 mm (i.d.) fused silica bonded-phase column (BPX70; SGE, Melbourne, Australia). The column oven was programmed from 125°C for 3 min to 220°C at 8°C/min with helium as carrier gas at a flow rate of 43 cm/s. Fatty acids were identified by comparison with standard mixtures of fatty acid methyl esters, and the results were calculated using response factors derived from chromatographing standards of known composition (Nu-Chek-Prep, Elysian, MN).

Statistical analysis. A single factor analysis of variance considered the effect of diet on retinal fatty acid content between the four groups. Post-hoc comparisons were performed using a Bonferroni/Dunn procedure. The *a priori* alpha level for both tests was 0.05.

RESULTS AND DISCUSSION

The retinal phospholipid fatty acid patterns are shown in Table 1. The four- and eight-week-old animals fed the safflower oil diet showed very low tissue levels of DHA (<3%) and substantial elevations in the 22:5n-6 levels (>21%): DHA/22:5n-6 ratios of <0.12. The commercial diet-fed animals had approximately the same levels of 22:5n-6 as DHA in their retinal lipids. The canola oil-fed animals had significantly higher levels of DHA than 22:5n-6 (ratio ≥2.9). The four- and eight-week-old fish oil-fed group had the highest levels of DHA, with DHA/22:5n-6 ratios of ≥54. The 22:5n-6 comprised less than 1% of the phospholipid fatty acids in the fish oil-fed group by comparison with values of 20% in the safflower group. The data for the brain for the four-week-old animals were similar to the retina, with the DHA/22:5n-6 ratios being 0.1, 0.9, 1.5, and 24 for the safflower, chow, canola, and fish oil diet groups, respectively.

TABLE 1
Fatty Acid Composition of Retinal Phospholipids from Guinea Pigs Fed Diets Containing Different Levels of n-3 PUFA[a]

	Age (four weeks)				Age (eight weeks)			
Fatty acid	Fish oil	Canola oil	Commercial	Safflower oil	Fish oil	Canola oil	Commercial	Safflower oil
16:0	19.5 ± 0.9[b]	18.3 ± 0.7[b]	20.2 ± 1.1	19.6 ± 1.0[b]	17.5 ± 0.4	17.9 ± 0.3	17.6 ± 0.4	17.6 ± 0.7
18:0	14.5 ± 1.7[c]	16.1 ± 0.8	18.4 ± 1.5	20.5 ± 2.1[c]	21.3 ± 1.2	21.0 ± 0.3	21.7 ± 1.0	21.2 ± 0.9
18:1	10.0 ± 0.6[c]	8.3 ± 0.3[c]	11.2 ± 0.4	9.0 ± 0.5[c]	11.0 ± 0.3	11.7 ± 0.4[b]	11.2 ± 0.5	9.3 ± 0.5[b]
18:2n-6	2.3 ± 0.3	2.4 ± 0.2	2.7 ± 1.4	2.7 ± 0.2	1.3 ± 0.2	1.6 ± 0.3[b]	1.3 ± 0.2	2.6 ± 0.4[b]
20:4n-6	8.5 ± 0.7[b]	11.1 ± 0.3	10.5 ± 1.6	9.1 ± 0.6	7.3 ± 0.4[b]	9.2 ± 0.3	9.6 ± 0.5	9.8 ± 0.3
20:5n-3	0.8 ± 0.2	—[d]	—	—	0.5 ± 0.1	—	—	—
22:4n-6	1.1 ± 0.1[c]	2.5 ± 0.1	2.7 ± 0.4	4.4 ± 0.3[c]	1.1 ± 0.1[c]	2.5 ± 0.1[c]	3.1 ± 0.2	4.5 ± 0.2[c]
22:5n-6	0.6 ± 0.06[c]	6.6 ± 0.9[c]	11.4 ± 2.5	20.6 ± 1.4[c]	0.5 ± 0.1[c]	5.2 ± 0.3[c]	14.1 ± 0.6	23.3 ± 1.8[c]
22:5n-3	1.8 ± 0.08[c]	1.4 ± 0.1[c]	1.0 ± 0.2	0.3 ± 0.03[c]	1.8 ± 0.2[c]	1.3 ± 0.1[c]	0.8 ± 0.1	0.2 ± 0.03[c]
22:6n-3	32.4 ± 0.4[c]	19.1 ± 1.5[c]	13.4 ± 1.0	2.4 ± 0.2[c]	30.8 ± 1.0[c]	21.0 ± 0.5[c]	12.4 ± 1.2	2.5 ± 0.8[c]
22:6/22:5n-6	54.0 ± 5.4	2.9 ± 0.4	1.2 ± 0.2	0.1 ± 0.01	61.6 ± 12.3	4.0 ± 0.2	0.9 ± 0.1	0.1 ± 0.04

[a]Mean ± standard deviation, n = 6 for four-week-old, n = 12 for eight-week-old animals; PUFA, polyunsaturated fatty acids.
[b,c]Significantly different from commercial diet fed values: [b]$P < 0.01$, [c]$P < 0.001$.
[d]Less than 0.1%.

These data show that guinea pigs raised on a diet containing α-linolenic acid (canola oil-based diet or the commercial guinea pig diet) have lower retinal and brain DHA levels relative to those fed fish oil. Furthermore, the group fed the safflower oil diet had very low DHA levels (<3% of the total fatty acids of retinal phospholipids). In rats and monkeys fed similar diets deficient in n-3 PUFA, the retinal DHA values do not fall below 40% of the value obtained by feeding control diets containing α-linolenic acid (soybean oil) (3–6). In the group fed the commercial guinea pig diet, the retinal and brain DHA/22:5n-6 ratios would be considered typical of tissue PUFA patterns induced by feeding n-3 PUFA-deficient diets in other species (rats and monkeys) (3–6,9).

It has been reported that rabbits fed chow diets have similar retinal PUFA profiles to those reported here for the guinea pigs fed the commercial diet (10). In rabbits fed rabbit chow containing linoleic and α-linolenic acid as the only dietary PUFA, the retinal tissue had approximately equal levels of 22:5n-6 and DHA up to nine weeks of age, and the high levels of 22:5n-6 were suppressed by the inclusion of fish oil in the diet (10). It was also noted that the retinal DHA/22:5n-6 ratio increased substantially in adult animals fed these diets from weaning. We have not examined the DHA/22:5n-6 ratios in adult guinea pigs.

The four diets used in this experiment contained four different levels of n-3 PUFA. As the level of dietary n-3 PUFA increased, the DHA levels in the retina and brain increased and the 22:5n-6 decreased. There was almost a complete absence of 22:5n-6 in the group fed fish oil, which suggests that DHA is a strong inhibitor of 22:5n-6 synthesis. An explanation as to why the guinea pigs fed α-linolenic acid do not have the same retinal DHA levels as those fed DHA would be that the guinea pig has a reduced capacity to synthesize long-chain PUFA from α-linolenic acid, relative to the rat and the rhesus monkey. Such a possibility is consistent with the finding of Willis (11) in which he reported that the guinea pig had a substantially lower Δ5 desaturase activity as compared with the rat. This is also supported by data showing a fourfold higher proportion of 20- and 22-carbon PUFA in the liver phospholipid fatty acids from rats fed canola oil diets as compared with guinea pigs fed canola oil diets (Sinclair, A.J., unpublished observations). The question of whether high levels of 22:5n-6 in the guinea pig retina are species-specific or associated with a functional deficit is presently being investigated in our laboratory.

ACKNOWLEDGMENTS

This research was supported by Wyeth Ayerst Research USA, Meadow Lea Australia, and Dano Chemo A/S Norway.

REFERENCES

1. Crawford, M.A., Casperd, N.M., and Sinclair, A.J. (1976) "The Long Chain Metabolites of Linoleic and Linolenic Acids in Liver and Brain in Herbivores and Carnivores," *Comp. Biochem. Physiol. 54B*, 395–401.
2. Sinclair, A.J. (1975) "Long Chain Polyunsaturated Fatty Acids in the Mammalian Brain," *Proc. Nutr. Soc. 34*, 287–291.
3. Connor, W.E., Neuringer, M., and Reisbick, S. (1992) "Essential Fatty Acids: The Importance of n-3 Fatty Acids in the Retina and Brain," *Nutr. Rev. 50*, 21–29.
4. Bourre, J.M., Francois, M., Youyou, A., Dumont, O., Piciotti, M., Pascal, G., and Durand, G. (1989) "The Effect of Dietary Alpha-Linolenic Acid on the Composition of Nerve Membranes, Enzymatic Activity, Amplitude of Electrophysiological Parameters, Resistance to Poisons and Performance of Learning Tasks in Rats," *J. Nutr. 119*, 1880–1892.
5. Watanabe, I., Kato, M., Aonuma, H., Hashimoto, A., Naito, Y., Moriuchi, A., and Okuyama, H. (1987) "Effect of Dietary Alpha-Linolenate/Linoleate Balance on the Lipid Composition and Electroretinographic Responses in Rats," *Adv. Biosci. 62*, 563–570.
6. Neuringer, M., Connor, W.E., Lin, D.S., Barstad, L., and Luck, S. (1986) "Biochemical and Functional Effects of Prenatal and Postnatal Omega-3 Fatty Acid Deficiency on Retina and Brain in Rhesus Monkeys," *Proc. Natl. Acad. Sci. 83*, 4021–4025.
7. Leat, W.M.F., Curtis, R., Millichamp, N.J., and Cow, R.W. (1986) "Retinal Function in Rats and Guinea Pigs Reared on Diets Low in Essential Fatty Acids and Supplemented with Linoleic or Linolenic Acids," *Ann. Nutr. Metab. 30*, 166–174.
8. Sinclair, A.J., O'Dea, K., Dunstan, G., Ireland, P.D., and Niall, M. (1987) "Effects on Plasma Lipids and Fatty Acid Composition of Very Low Fat Diets Enriched with Fish or Kangaroo Meat," *Lipids 22*, 523–529.
9. Fiennes, R.N.T-W., Sinclair, A.J., and Crawford, M.A. (1973) "Essential Fatty Acid Studies in Primates: Alpha-Linolenic Acid Requirements of Capuchins," *J. Med. Primatology 2*, 155–169.
10. Lin, D.S., Anderson, G.J., and Connor, W.E. (1991) "High Levels of the (n-6) Fatty Acid 4,7,10,13,16-Docosapentaenoic Acid in the Retinas of Rabbits Are Reduced by Feeding Dietary Fish Oil from Birth to Adult Live," *J. Nutr. 121*, 1924–1931.
11. Willis, A.L. (1981) "Unanswered Questions in Essential Fatty Acid and Prostaglandin Research," *Prog. Lipid Res. 20*, 839–850.

[Received June 28, 1994, and in revised form February 13, 1995; Revision accepted February 23, 1995]

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) "Temperature and Diet Effects on Trout," *J. Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) "Temperature and Diet Effects on Trout," in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., "Temperature and Diet Effects on Trout," *J. Biol. Chem.*, in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

AVAILABILITY OF FISH OIL TEST MATERIALS

The Fish Oil Test Materials Program is a cooperative effort of the National Institutes of Health (NIH) and the National Oceanic and Atmospheric Administration/Department of Commerce (NOAA/DOC). This program provides a long-term, consistent supply of quality-assured/quality-controlled test materials to researchers that facilitates the evaluation of ω-3 fatty acids in health and disease.

Current test materials available, in both capsules and bulk, include: (i) vacuum deodorized menhaden oil; (ii) ethyl esters of olive, corn, and safflower oils; (iii) commercial preparations of corn, olive, and safflower oil; (iv) n-3 ethyl ester concentrates; (v) EPA ethyl ester; and (vi) DHA ethyl ester.

To qualify to receive the fish oil test materials from the Program, the researcher must fill out an application. The applicant must: (i) have peer-reviewed research indicating the need for the requested materials, and (ii) submit a cor-

(continued on next page)

rectly completed application form and a signed waiver of liability. *The awarded materials are provided free of charge (researchers will pay shipping costs).*

Investigators may obtain further information and an application form from Fish Oil Test Materials Program, NIAAA, National Institutes of Health, 12501 Washington Ave., Flow Building, Room 2, Rockville, MD 20852 (Phone: Beth Aurecchia, 301-443-9643; Fax: 301-594-0035).

CALENDAR

June 1995

2nd International Congress of the International Society for the Study of Fatty Acids and Lipids, June 7–10, National Institutes of Health, Bethesda, Maryland, USA. Information: A.P. Simopolous, ISSFAL, 2001 S St., N.W., Suite 530, Washington, DC 20009 USA.

International Lecture Evening, SCI Oils and Fats Group, June 8, 1995, SCI Building, 14/15 Belgrave Sq., London, England. Prof. Karé Larsson to speak on "The Physical Properties of Lipids in Relation to Their Function in Foods." Information: Conference Secretariat, SCI, 14/15 Belgrave Sq., London SW1X 8PS, United Kingdom (Fax: 44-71-823-1698).

18th Nordic Lipid Symposium, June 18–21, Reykjavik, Iceland. Information: G. Lambertsen, Lipidforum Secretariat, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-325724).

August 1995

36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September 1995

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October 1995

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

American College of Nutrition 36th Annual Meeting, Oct. 13–15, 1995, Omni Shoreham Hotel, Washington, DC. Information: American College of Nutrition, c/o Hospital for Joint Disease, 301 E. 17th St., New York, NY 10003 (Fax: 212-777-1103).

Lipidforum Seminar: Lipids in Animal Nutrition, Oct. 25–26, 1995, Research Centre Foulum, Tjele (Viborg), Denmark. Information: G. Lambertsen, Lipidforum, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-32 57 24).

November 1995

XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

February 1996

Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Hennincksstraat, B-2610 Wilrijk (Antwerpen), Belgium (Phone/Fax: 32 (3)828.89.61).

Determination of Lysine Modification Product ε-*N*-Pyrrolylnorleucine in Hydrolyzed Proteins and Trout Muscle Microsomes by Micellar Electrokinetic Capillary Chromatography

Rosario Zamora*, José L. Navarro and Francisco J. Hidalgo

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla, Spain

ABSTRACT: ε-*N*-Pyrrolylnorleucine (Pnl) is a product of the reaction between the lipid peroxidation product 4,5(*E*)-epoxy-2(*E*)-heptenal (EH) and the ε-amino group of lysine. Because Pnl might also be produced in proteins, a specific method to determine this compound in protein hydrolysates has been developed. Homoarginine, added as the internal standard, and Pnl are derivatized with diethyl ethoxymethylenemalonate and analyzed by micellar electrokinetic capillary chromatography. The method also analyzes lysine and arginine, and these analyses were useful in determining losses of these amino acids after treatment with EH. The lowest concentration of Pnl detected with acceptable reproducibility is 5 nmol/mL, and the coefficient of variation was determined from four standard curves assayed on separate days. Detector response was linear for samples containing 1.6 to 74 nmol/mL of Pnl. The assay was applied in investigations of Pnl production in bovine serum albumin (BSA) and trout muscle microsomes treated with EH. When BSA was incubated overnight with 30 mM EH, 76% of lysine residues were modified, and a part of these residues were detected as Pnl (12%). Pnl formation was also detected when trout muscle microsomes were incubated for three hours with 1 or 10 mM EH. These results show that Pnl is produced *in vitro* in proteins treated with the lipid peroxidation product EH, and suggest that Pnl might also be constituent of *in vivo* damaged proteins by their reaction with oxidized lipids.

Lipids 30, 477–483 (1995).

Lipid peroxidation seems to be a consequence of *in vivo* oxidative stress, and has been implicated as a deleterious factor in a variety of diseases, including carcinogenesis, mutagenesis, aging, and atherosclerosis (1–3). Although the mode of action is not clear, the production of reactive carbonyl compounds and their subsequent reactions with cellular nucleophiles may be one of the mechanisms (4,5). These reactive carbonyl compounds are strong electrophiles and are capable of reacting rapidly with nucleophiles, including amino acids and proteins. The actual carbonyl compounds involved in such reactions with cellular nucleophiles are not yet well known (6), which is due at least in part to a lack of satisfactory analytical methods to study these reactive carbonyl compounds or their reaction products.

Previous work from this laboratory has shown that the lipid peroxidation product 4,5(*E*)-epoxy-2(*E*)-heptenal (EH) can react with amines, amino acids, and proteins to produce brown-colored and fluorescent products (7,8). The first step in this complex polymerization reaction is the formation of both 1-substituted-2-(1′-hydroxyalkyl)pyrroles and 1-substituted-pyrroles. Pyrroles with such structures have been isolated and characterized in the reaction between EH and butylamine, glycine methyl ester and lysine (9,10). 1-Substituted-2-(1′-hydroxyalkyl)pyrroles can later polymerize to produce brown macromolecules with fluorescent lipofuscin-like characteristics (9). In contrast, 1-substituted-pyrroles seem to be final products in these reactions, and their presence in proteins might be related to damage of proteins as a consequence of its reaction with oxidized lipids.

The objective of this study was to develop an analytical procedure for the isolation and quantitation of the interaction product ε-*N*-pyrrolylnorleucine (Pnl) [This compound was previously named 1-(5′-amino-5′-carboxypentyl)pyrrole (see Ref. 10). ε-*N*-Pyrrolylnorleucine is preferred here because it better reflects the amino acid character. As an abbreviation, a three-letter code has been chosen.] (Fig. 1A). Pnl is the 1-substituted-pyrrole produced in the reaction between EH and the ε-amino group of lysine (10), and might be produced in the presence of proteins. Determination of Pnl may be a satisfactory analytical tool for studying protein damage due to oxidative stress.

EXPERIMENTAL PROCEDURES

EH was prepared from 2(*E*),4(*E*)-heptadienal as described by Swoboda and Peers (11). 2(*E*),4(*E*)-Heptadienal, L-lysine

*To whom correspondence should be addressed at Instituto de la Grasa, Avenida Padre García Tejero, 4, 41012 Sevilla, Spain.

Abbreviations: BSA, bovine serum albumin; DEEMM, diethyl ethoxymethylenemalonate; EH, 4,5(*E*)-epoxy-2(*E*)-heptenal; HPLC, high-performance liquid chromatography; IS, internal standard; KRPB, Krebs-Ringer phosphate buffer; MECC, micellar electrokinetic capillary chromatography; MS, mass spectra; NMR, nuclear magnetic resonance; Pnl, ε-*N*-pyrrolylnorleucine, 1-(5′-amino-5′-carboxypentyl)pyrrole; UV, ultraviolet.

Copyright © 1995 by AOCS Press

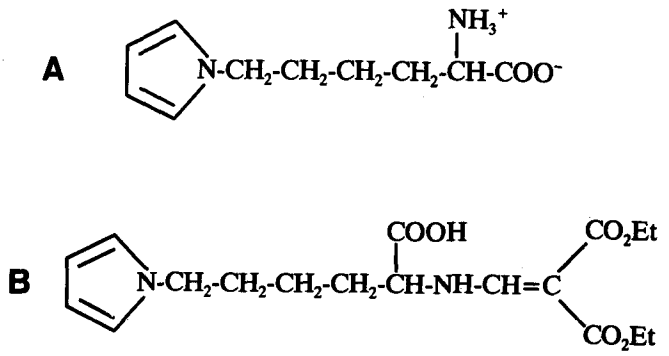


FIG. 1. Chemical structures of ε-*N*-pyrrolylnorleucine (A) and its *N*-[2,2-*bis*(ethoxycarbonyl)vinyl] derivative (B).

monohydrochloride, 3-chloroperoxybenzoic acid, and diethyl ethoxymethylenemalonate (DEEMM) were purchased from Aldrich Chemical (Milwaukee, WI). All other chemicals used were analytical-grade and were purchased from reliable commercial sources. ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) spectra at 300 and 75.4 MHz, respectively, were determined on a Bruker AC-300P (Karlsruhe, Germany), with sodium 3-(trimethylsilyl)-1-propanesulfonate as internal standard. Two-dimensional NMR was used for the assignment of ^{13}C NMR spectra. Mass spectra (MS) were obtained in an AEI-MS-30SB-VG mass spectrometer (VG Analytical, Manchester, United Kingdom).

Synthesis of Pnl. Pnl was prepared by a slight modification of a previously described procedure (10). Briefly, an emulsion of EH (0.5 mmol) in 4 mL of 0.3 M sodium borate, pH 9.0, was treated with lysine (1 mmol) and stirred overnight at 37°C. The reaction mixture was fractionated by semipreparative high-performance liquid chromatography (HPLC) on a Spherisorb ODS2 C18 column (25 × 1 cm) and eluted with 5% acetonitrile in deionized water at 40°C. The HPLC system consisted of a 126 Programmable Delivery Module and a 168 Diode Array Detector Module (Beckman, Fullerton, CA). Data acquisition and processing were effected with software System Gold 7.1 version (Beckman). The flow rate was 5 mL/min with detection by absorbance at 250 nm. A loop of 500 µL was used in all the experiments. Under these conditions, Pnl eluted at 9.5 min. After 10 min, the column was cleaned with acetonitrile and equilibrated for 10 min with the solvent before the next injection. Pnl was collected in successive injections, and combined. Solutions were gassed with nitrogen to remove acetonitrile, and freeze-dried to obtain chromatographically pure Pnl.

Derivatization of Pnl and other amino acids with DEEMM. The free amino groups in Pnl and other amino acids obtained in protein hydrolysates were protected by using DEEMM, which facilitated separation and detection. The derivatization reaction was carried out by modification of a previously described procedure (12), as follows: a mixture of standard amino acids, or a neutralized protein hydrolysate (50 µL containing approximately 0.5 mg of the pool of amino acids), was dissolved in 1 M sodium borate buffer (pH 9.0) (3 mL) containing 0.02% of sodium azide, then mixed with 100 µL of internal standard (IS) solution (homoarginine in deionized water, 1 mg/mL), and finally treated with 5 µL of DEEMM. The reaction mixture was stirred at 50°C for 60 min to completely derivatize proline, the less reactive amino acid for this reaction (12).

Detection of Pnl in amino acid mixtures. Two different techniques (HPLC and capillary electrophoresis) were used for detection and quantitation of Pnl, and other amino acids, in amino acid mixtures. The HPLC system (Waters, Milford, MA) consisted of a Model 600E multi-solvent delivery system, a Wisp Model 712 automatic injector, and a Model 484 ultraviolet (UV)-visible detector. Pnl was derivatized and separated on a 300 × 3.9 mm i.d. reversed-phase column (Nova-Pack C18, 4 µm; Waters) using a previously described gradient for amino acid analysis (13). The capillary electrophoresis system consisted of a Beckman 2100 P/ACE unit equipped with a UV detector. (The P/ACE unit was linked to a 386/33 computer loaded with System Gold Software for data collection and handling.) The capillary consisted of 50 cm in length uncoated silica tubing with 75 µm internal diameter and a detector aperture window of 100 × 200 µm. The sample was introduced into the capillary by pressure injection for 2.0 s. Micellar electrokinetic capillary chromatography (MECC) was carried out at 30 kV and 30°C in 100 mM sodium borate buffer, pH 8.4, containing 100 mM SDS. Electrophoretic profiles were monitored by UV absorption at 280 nm. After repetitive runs, a decrease in the resolution of the capillaries was sometimes observed and the capillary was regenerated by washing with 15% Extran solution (Merck, Darmstadt, Germany) for 10 min at 35°C, and then with water for another 10 min.

Stability of Pnl during hydrolysis. Both acid and basic hydrolyses were tested to obtain the best Pnl recovery in protein hydrolysates. Acid hydrolysis was carried out by dissolving Pnl in 6 M hydrochloric acid, gassing the solutions with nitrogen, and sealing in hydrolysis tubes under nitrogen. Samples were incubated in an oven at 120°C for 18 h. Analogous treatment was used for alkaline hydrolysis, with 2 M sodium hydroxide instead of 6 M hydrochloric acid.

Quantitation. Standard plots of peak are ratio of Pnl, arginine, and lysine, to the IS vs. their concentrations were run on a daily basis. Linear least squares regression was performed to determine the slope, y-intercept, and correlation coefficient of the best fit line. Amino acid concentrations in unknown samples were calculated using results of the regression analysis.

Precision of the method and determination of the lower limit of quantitation. Precision and accuracy of the assay were determined by analyzing the back-calculated sample concentrations and regression parameters from four standard curves of Pnl, arginine, and lysine prepared and analyzed on separate days. Coefficients of variation of mean-predicted concentration for independently assayed standard mixtures pro-

vided the measure of precision. The lower limit of quantitation was defined as the minimum concentration of amino acid that can be analyzed with a coefficient of variation not exceeding 15%.

Determination of Pnl in protein hydrolysates. Bovine serum albumin (BSA, 10 mg) was dissolved in 1 mL of 50 mM sodium phosphate buffer pH 7.0 (or sodium borate buffer pH 9.0) and treated with EH (4 mg). The mixture was allowed to react for 24 h at 37°C. Fifty microliters of the browned protein solution were then mixed with 800 µL of 2 M NaOH and hydrolyzed by heating under nitrogen for 18 h at 120°C. The resulting solution was neutralized with hydrochloric acid, filtered, and derivatized with DEEMM. Twenty-five microliters of protected amino acids were diluted with 125 µL of water and submitted to MECC as previously described.

Determination of Pnl in trout muscle microsomes incubated with EH. Muscle microsomes from freshly killed rainbow trout (*Salmo gairdnerii*) were prepared by differential centrifugation using a modification of the procedure described by McDonald *et al.* (14). Trout muscle was homogenized in 4 vol of cold Krebs-Ringer phosphate buffer (KRPB), pH 7.4 (15), using an Omni 2000 blender (Omni Int'l, Waterbury, CT) at the maximum speed for 20 s. Fish homogenate was then centrifuged at 22,000 × *g* and 4°C for 30 min to remove mitochondria and fractions heavier than mitochondria. The post-mitochondrial supernatant was collected and centrifuged at 105,000 × *g* and 4°C for 60 min. The microsomal pellet was collected and washed twice by suspending the microsomes in cold KRPB (pH 7.4) and by ultracentrifugation under the same conditions. Washed microsomes were dispersed in KRPB, and protein concentration, measured according to Bradford method (16), was adjusted to 3.0 mg/mL for experiments.

Six hundred and seventy microliters of the microsomal preparation were diluted with 3 mL of KRPB and incubated at 37°C for 3 h in the presence, or not (control), of 1 and 10 mM EH. At the end of the incubation period, samples were centrifuged at 105,000 × *g* and 4°C for 60 min. The microsomal pellet was collected and washed with water. Washed incubated microsomes were dispersed in water (1.8 mL), treated with 0.9 mL of 6 M NaOH, and hydrolyzed by heating under nitrogen for 18 h at 120°C. The resulting solution was neutralized with hydrochloric acid, filtered, and derivatized with DEEMM. Twenty-five microliters of protected amino acids were diluted with 125 µL of water and submitted to MECC as previously described.

RESULTS

Preparation and derivatization of Pnl. Pnl is one of the products of the reaction between EH and lysine. The method developed in this study for its isolation shortens the time previously described by cleaning the column after eluting Pnl. Pnl was obtained chromatographically pure, and its ^{1}H and ^{13}C NMR and MS confirmed the proposed structure (10).

The derivatization reaction with DEEMM is quick and clean, producing the corresponding *N*-[2,2-*bis*(ethoxycarbonyl)vinyl] derivative (Fig. 1B). This derivative provided a high absorptivity for easy detection at 280 nm (12). Its HPLC (data not shown) and MECC profiles exhibited one peak for Pnl, which could be used for its separation and quantification only in MECC. Figure 2A shows the electropherogram obtained when derivatized Pnl was submitted to MECC. Peak 5 at 4.20 min corresponded to Pnl. The IS (peak 9) appeared at 6.41 min. The peak in the high-performance liquid chromatogram overlapped with that of lysine and could not be separated from it.

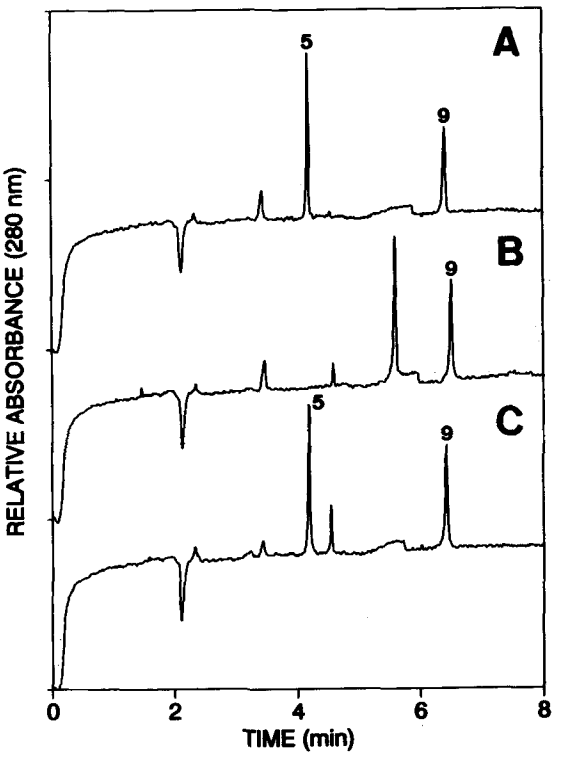


FIG. 2. Effect of hydrolysis on ε-N-pyrrolylnorleucine (Pnl, Peak 5). A, Pnl before hydrolysis. B, Pnl after acid hydrolysis. C, Pnl after basic hydrolysis. Peak 9 is internal standard, homoarginine.

Stability of Pnl during hydrolysis. Because Pnl might be produced in proteins by reaction of the ε-amino group of lysine residue with the lipid peroxidation product EH, the method for its detection and quantification should include the hydrolysis of the protein as a first step. Figure 2 shows electropherograms of derivatized Pnl before hydrolysis (A), after acid hydrolysis (B), and after basic hydrolysis (C). As indicated in Figure 2B, the peak due to Pnl disappeared after acid hydrolysis. The pyrrole ring in Pnl is apparently very sensitive to acid condition, but is resistant to basic hydrolysis (Fig. 2C). For this reason, only basic hydrolysis was used in the rest of this study. Some attempts to recover Pnl from proteins by enzymatic hydrolysis were also tested, but they were not satisfactory.

Detection of Pnl in amino acid mixtures. Twenty-two common amino acids and Pnl were hydrolyzed and analyzed by MECC. For the determination of Pnl, mixtures of amino acids

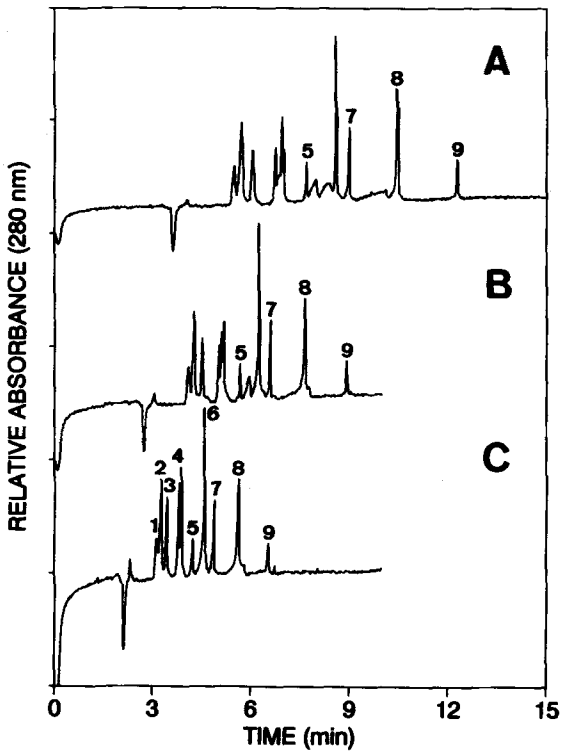


FIG. 3. Effect of voltage on peak resolution of ε-*N*-pyrrolylnorleucine (Peak 5). A, 20 kV; B, 25 kV; C, 30 kV.

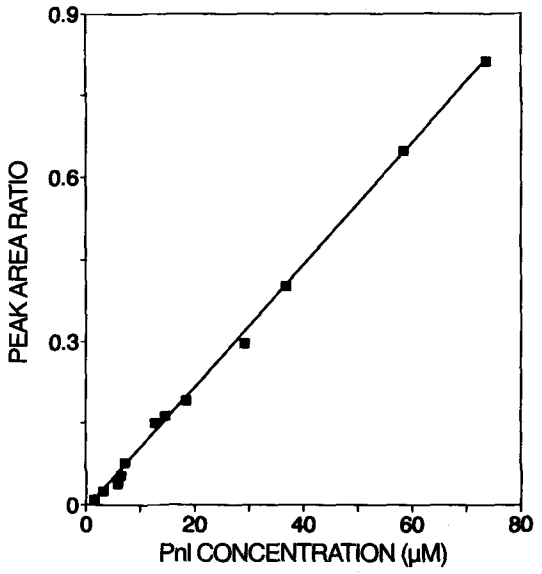


FIG. 4. Standard curve of ε-*N*-pyrrolylnorleucine obtained by using twelve standard mixtures independently prepared and assayed on four different days.

were used to optimize electrophoretic conditions. Figure 3 shows the influence of the voltage on the separation of the peak corresponding to Pnl (peak 5). A voltage of 30 kV produced the best and most rapid separation of Pnl (Fig. 3C). Also, a concentration of 100 mM SDS was required for the best separation. Assignment of the peaks in the electrophoretic pattern in Figure 3C obtained with mixtures of different amino acids were as follows: Peak 1 corresponded to histidine, serine, and threonine. Peak 2 corresponded to alanine, aspartic acid, glycine, proline, serine, threonine, and tyrosine. Peak 3 was common for most amino acids: alanine, ammonia, arginine, asparagine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, Pnl, proline, serine, threonine, tyrosine, and valine. Peak 4 corresponded to cysteine, cystine, isoleucine, leucine, phenylalanine, and tryptophan. Peak 5 corresponded to Pnl. Peak 6 appeared as one or two components depending on the amino acid composition of the protein mixture (for example, two peaks were observed with a hydrolysate of BSA, Fig. 5A). This peak (or two peaks) corresponded to ammonia, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, threonine and tryptophan. Peak 7 corresponded to arginine. Peak 8 corresponded to lysine. Peak 9 corresponded to the IS homoarginine. Therefore, Peak 5 (Pnl), Peak 7 (Arg), Peak 8 (Lys), and Peak 9 (homoarginine) were the only peaks that corresponded to single amino acids and could be used for quantification.

Assay validation. Figure 4 shows a typical calibration curve of Pnl in basic hydrolysates of amino acids. For twelve standard mixtures, ranging from 1.59 to 73.7 nmol/mL, the Pnl (and also arginine and lysine) to IS electrophoretic peak area ratio was directly proportional to amino acid concentration. Values of the slope, y-intercept, and correlation coefficient of the calibration curves obtained for the three amino acids are shown in Table 1. Coefficients of variation for the mean predicted Pnl concentration of each standard mixture were equal or less than 15% from 5.84 to 73.7 μM (Table 2). Pnl was readily detected in samples at a concentration below 5.84 μM; however, the variability in peak response was considered unacceptable, and a concentration of 5.84 μM was considered the practical lower limit of quantitation. Relative recoveries of Pnl ranged from 90 to 108% within the 6–74 μM range (Table 2).

Determination of Pnl in BSA incubated in the presence of EH. BSA was incubated with 30 mM EH for 24 h, and then subsequently hydrolyzed, derivatized, and analyzed by MECC. Figure 5 shows the electropherograms obtained with starting BSA (A), BSA/EH mixture incubated at pH 7.0 (B), and BSA/EH mixture incubated at pH 9.0 (C). The incubation of BSA with EH produced Pnl (Peak 5), and this corresponded to a decrease in the peak area of lysine (Peak 8). Reaction of EH with the ε-amino group of lysine produces, in addition to Pnl, 1-(5′-amino-5′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole and macromolecular fluorescent substances by polymerization of this latter compound (10). The alkaline hydrolysis of 1-(5′-amino-5′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole yielded a decomposition product that appeared under Peak 6 (data not shown). Possibly for this reason, electropherograms 5B and 5C also showed a change in Peak 6. At higher pH, the BSA/EH reaction mixture produced more Pnl, which also corresponded to a decrease in the peak area of lysine, suggesting that Pnl formation was favored at basic pH. Analogous results were obtained when the EH/lysine reaction mixture

TABLE 1
Calibration Curves Obtained for Pnl, Arginine (Arg), and Lysine (Lys), Using Twelve Standard Mixtures[a]

Amino acid	Slope	y-Intercept	Correlation coefficient
Pnl	0.0112	−0.012	0.999
Arg	0.0397	−0.102	0.994
Lys	0.0466	−0.028	0.992

[a]Standard mixtures were independently prepared and assayed on four different days. Values used for calibration curves were the mean of these four measurements; Pnl, ε-N-pyrrolylnorleucine, 1-(5'-amino-5'-carboxypentyl)pyrrole.

TABLE 2
Relative Recovery and Reproducibility of the Analytical Method for the Quantitation of Pnl Using MECC[a]

Amount added (nmol/mL)	Mean amount found (nmol/mL)	Relative recovery (%)	Coefficient of variation (%)
1.59	1.84	116	35
3.19	3.28	103	25
5.84	5.26	90	15
6.38	5.83	91	15
7.37	7.93	108	13
12.8	13.8	108	11
14.6	15.6	107	13
18.4	18.0	98	12
29.2	27.6	94	6
36.8	36.7	100	7
58.4	58.9	101	7
73.7	75.1	102	8

[a]The number of replicates was four for each amount added. Relative recoveries and coefficients of variation were calculated for the whole process, including hydrolysis and derivatization. MECC, micellar electrokinetic capillary chromatography. See Table 1 for other abbreviation.

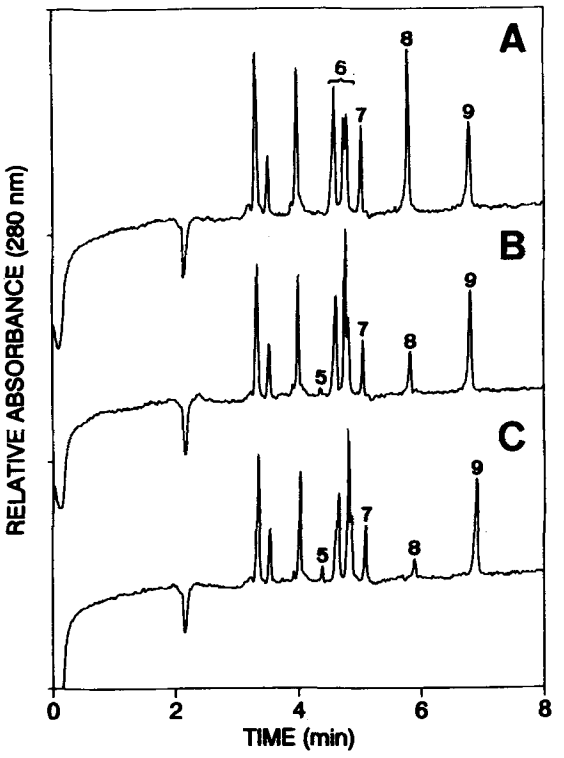


FIG. 5. Electropherograms obtained for amino acid analysis after basic hydrolysis and derivatization of nonincubated bovine serum albumin (A), bovine serum albumin incubated overnight with 4,5(*E*)-epoxy-2(*E*)-heptenal at pH 7.0 (B), and bovine serum albumin incubated overnight with 4,5(*E*)-epoxy-2(E)-heptenal at pH 9.0 (C). ε-*N*-Pyrrolylnorleucine is Peak 5.

without derivatization was fractionated by analytical HPLC (17).

Table 3 shows the Pnl, arginine, and lysine concentrations in hydrolyzed BSA and BSA incubated with EH at pH 7.0. After 24 h incubation, 76% of lysine residues were modified, and approximately 12% of lysine residues were detected as Pnl. The other 64% of modified lysine residues are in part responsible for the changes in Peak 6 (Fig. 5B). A decrease of 34% of arginine residues was also observed by MECC.

For comparison purposes, the loss of amino acids in BSA incubated with EH at pH 7.0 was also determined by HPLC, using a previously described gradient (13) after acid hydrolysis (Table 4). Amino acid composition determined in this way showed some differences in respect to the data found by MECC. First, a smaller decrease in arginine concentration was observed, suggesting that arginine may react with EH to produce a complex or derivative stable to basic hydrolysis but it is partly reversible by acid hydrolysis. In addition, a lower decrease in lysine was also observed that may be due to both a lysine release during acid hydrolysis of some of the lysine/EH products, and the appearance of other derivatives (like Pnl) with the same retention as lysine. Finally, an important decrease in histidine was also observed by HPLC. This could not be corroborated by MECC because histidine appears in Peak 1 and Peak 3, and its concentration may be masked by other amino acids.

TABLE 3
Determination of Pnl, Arg, and Lys in BSA and BSA Incubated with EH at pH 7.0[a]

Amino acid	BSA	BSA/EH	Difference (%)
Pnl	—	1.03 ± 0.06	—
Arg	3.33 ± 0.12	2.20 ± 0.18	−34
Lys	8.39 ± 0.37	2.01 ± 0.23	−76

[a]Values are expressed in μmol of amino acid/mL incubation mixture and represent the mean ± *SD* of four incubation mixtures. Amino acid analysis was determined by MECC after basic hydrolysis and derivatization with diethyl ethoxymethylenemalonate; BSA, bovine serum albumin; EH, 4,5(*E*)-epoxy-2(*E*)-heptenal. See Tables 1 and 2 for other abbreviation.

Determination of Pnl in trout muscle microsomes incubated with EH. Trout muscle microsomes were incubated with 1 or 10 mM EH for 3 h at 37°C, and then subsequently hydrolyzed, derivatized, and analyzed by MECC. Figure 6 shows the electropherograms obtained with control microsomes (A), microsomes incubated with 1 mM EH (B), and microsomes incubated with 10 mM EH (C). Analogously to BSA, the incubation of microsomes with EH produced Pnl (Peak 5), and this corresponded to a decrease in the peak area of lysine. Table 5 shows the Pnl, arginine, and lysine concen-

TABLE 4
Amino Acid Composition of BSA and BSA Incubated with EH at pH 7.0[a]

Amino acid	BSA	BSA/EH	Difference (%)
Aspartic acid	7.48	7.63	+2
Glutamic acid	11.57	11.34	−2
Serine	4.21	4.29	+2
Histidine	2.65	1.88	−29
Glycine	2.45	2.46	0
Threonine	5.11	5.21	+2
Arginine	3.53	2.97	−16
Alanine	6.88	6.90	0
Proline	4.02	3.78	−6
Tyrosine	2.84	2.73	−4
Valine	5.26	5.31	+1
Methionine	0.64	0.65	+2
Cystine	2.55	2.60	+2
Isoleucine	2.18	2.19	0
Leucine	9.10	9.28	+2
Phenylalanine	4.16	4.12	−1
Lysine	8.84	4.86	−45

[a]Values are expressed in μmol of amino acid/mL incubation mixture and represent the mean of two independent incubation mixtures. Amino acid analysis was determined by high-performance liquid chromatography. See Table 3 for other abbreviations.

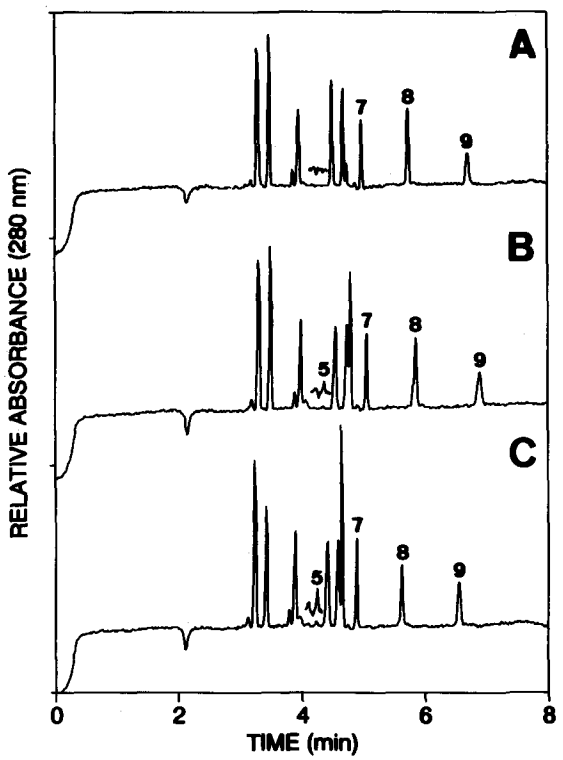


FIG. 6. Electropherograms obtained for amino acid analysis after basic hydrolysis and derivatization of untreated trout microsomes (A), trout microsomes incubated for 3 h with 1 mM 4,5(*E*)-epoxy-2(*E*)-heptenal (B), and trout microsomes incubated for 3 h with 10 mM 4,5(*E*)-epoxy-2(*E*)-heptenal (C). ε-*N*-Pyrrolylnorleucine is Peak 5.

trations determined by MECC. After 3-h incubation in the presence of 1 mM EH, 20% of lysine residues in microsomal proteins were modified and approximately 6% of lysine residues were detected as Pnl. These percentages increased when the incubation was carried out in the presence of a higher concentration of EH. Thus, after 3-h incubation, 34% of lysine residues in microsomal proteins were modified and approximately 11% of lysine residues were detected as Pnl. Analogously to BSA, some changes were also observed in Peak 6 (Fig. 6). However, no changes in arginine residue were observed.

TABLE 5
Determination of Pnl, Arg, and Lys in Trout Muscle Microsomes Incubated for 3 h at 37°C in the Presence, or Not (control), of 1 and 10 mM EH[a]

Amino acid	Control	1 mM EH	10 mM EH
Pnl	—	6.1 ± 2.3	10.3 ± 1.4
Arg	65.3 ± 3.7	64.5 ± 1.3	67.5 ± 1.8
Lys	69.3 ± 3.9	55.8 ± 1.2	45.6 ± 3.1

[a]Values are expressed in ng of amino acid/μg of protein, and represent the mean ± SD of five experiments. Amino acid analysis was determined by MECC after basic hydrolysis and derivatization with diethyl ethoxymethylenemalonate. See Tables 1, 2, and 3 for abbreviations.

DISCUSSION

Analogously to its reaction with lysine (10), the results obtained in this study show that EH reacts with protein amino groups, producing a decrease in the basic amino acids recovered after acid or basic hydrolysis. A part of lysine residues were transformed into Pnl, a new amino acid that was previously characterized in the reaction between EH and lysine (10). This study proposes a method to determine this amino acid that can be applied to proteins damaged by their reaction with oxidized lipids. The method is based on the basic hydrolysis of the protein and derivatization of the resulting amino acids with DEEMM. This analysis by capillary electrophoresis takes only 8 min, and Pnl can be easily detected and quantified.

The method has been applied to the study of the *in vitro* damage suffered by trout muscle microsomes and BSA incubated for different times and in the presence of several concentrations of EH. The results obtained show that the decrease in lysine recovered after basic hydrolysis depended on the concentration of EH and the incubation time. Thus incubation of trout muscle microsomes for 3 h at 37°C in the presence of 1 mM EH decreased by 20% the lysine resides recovered after basic hydrolysis, and this loss increased to 34% when the reaction was carried out in the presence of 10 mM EH. The proportion of lysine residues that were transformed into Pnl was constant at the two concentrations assayed. About 30% of these modified lysine residues were detected as Pnl. Part of the other 70% might have been converted, in a first step, into 1-(5′-amino-5′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole, and, lately, into the macromolecular fluorescent substances derived from polymerization of this latter compound (9,10).

When the study was carried out with BSA using 30 mM EH and 24-h incubation, the percentage of modified lysine residues increased to 76%. However, the percentage of these

residues that was detected as Pnl decreased to 16%, suggesting that very high concentrations of EH or very long incubation times, favored reactions other than Pnl formation. These results are in agreement with previous studies on EH/lysine reaction mechanism (17).

Differently to lysine, arginine residues recovered after basic hydrolysis were unmodified in the treatments assayed with muscle microsomes, but not when the reaction was carried out with BSA, suggesting that modification of this amino acid is more difficult than modification of lysine, and higher incubation times or higher concentrations of EH are needed.

In spite of the importance given to the problem of *in vivo* oxidative stress, which has been related to the development of several chronic and acute diseases, no absolute and definitive index has yet been discovered (18). This is probably due to the complexity of the problem. It involves many different processes and it is very difficult to find only one biomarker that is related to all these processes. One approximation to this problem may be the measurement of one of the products from one of the processes involved, and to try to demonstrate that a change in this measurement is related to a change in the overall oxidative stress, or with the development of the related diseases. Thus, for example, the measurement of volatile pentane or ethane has been proposed, among others (19,20).

The results obtained in this study propose a method to determine an amino acid that may be produced during oxidative stress. It is based on Pnl, an amino acid that has been shown to be produced in BSA and trout microsomes when free amino groups of their lysine residues react with the lipid peroxidation product EH, and, therefore, their formation might be related to the conditions that produce lipid peroxidation. Additional studies are needed to detect the presence of Pnl in *in vivo* oxidatively-modified proteins, and to establish a relationship, if any, between Pnl concentration and oxidative stress.

ACKNOWLEDGMENTS

This study was supported in part by the Comisión Interministerial de Ciencia y Tecnología of Spain (Projects ALI91-0409 and ALI94-0763) and the Junta de Andalucía (Project 2075).

REFERENCES

1. Tappel, A.L. (1973) Lipid Peroxidation Damage to Cell Components, *Fed. Proc.* 32, 1870–1874.
2. Rice-Evans, C., and Burdon, R. (1993) Free Radical-Lipid Interactions and Their Pathological Consequences, *Prog. Lipid Res. 32*, 71–110.
3. Halliwell, B., and Chirico, S. (1993) Lipid Peroxidation: Its Mechanism, Measurement, and Significance, *Am. J. Clin. Nutr. 57*, 715S–725S.
4. Esterbauer, H., Schaur, R.J., and Zollner, H. (1991) Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde and Related Aldehydes, *Free Rad. Biol. Med. 11*, 81–128.
5. Steinberg, D. (1991) Lipoprotein Modification and Atherogenesis, *Atherosclerosis Rev. 23*, 115–121.
6. Ebeler, S.E., Shibamoto, T., and Osawa, R. (1994) in *Lipid Chromatographic Analysis*, (Shibamoto, T., ed.) pp. 223–249, Marcel Dekker, New York.
7. Zamora, R., and Hidalgo, F.J. (1992) Browning and Fluorescence Development During Microwave Irradiation of a Lysine/(*E*)-4,5-epoxy-(*E*)-2-heptenal Model System, *J. Agric. Food Chem. 40*, 2269–2273.
8. Hidalgo, F.J., and Zamora, R. (1993) Non-Enzymatic Browning and Fluorescence Development in a (*E*)-4,5-epoxy-(*E*)-2-heptenal/Lysine Model System, *J. Food Sci. 58*, 667–670.
9. Hidalgo, F.J., and Zamora, R. (1993) Fluorescent Pyrrole Products from Carbonyl-Amine Reactions, *J. Biol. Chem. 268*, 16190–16197.
10. Zamora, R., and Hidalgo, F.J. (1994) Modification of Lysine Amino Groups by the Lipid Peroxidation Product 4,5(*E*)-Epoxy-2(*E*)-heptenal, *Lipids 29*, 243–249.
11. Swoboda, P.A.T., and Peers, K.E. (1978) *trans*-4,5-Epoxyhept-*trans*-2-enal. The Major Volatile Compound Formed by the Copper and Alpha-Tocopherol Induced Oxidation of Butterfat, *J. Sci. Food Agric. 29*, 803–807.
12. Alaiz, M., Giron, J., Hidalgo, F.J., Maza, M.P., Millan, F., Zamora, R., and Vioque, E. (1989) Esterification of Amino Acids as Their 2,2-*bis*(ethoxycarbonyl) Vinyl Derivatives, *Synthesis*, 544–547.
13. Alaiz, M., Navarro, J.L., Giron, J., and Vioque, E. (1992) Amino Acid Analysis by High-Performance Liquid Chromatography After Derivatization with Diethyl Ethoxymethylenemalonate, *J. Chromatogr. 591*, 181–186.
14. McDonald, R.E., Kelleher, S.D., and Hultin, H.O. (1979) Membrane Lipid Oxidation in a Microsomal Fraction of Red Hake Muscle, *J. Food Biochem. 3*, 125–134.
15. De Luca, H.F. (1972) in *Manometric and Biochemical Techniques* (Umbreit, W.W., Burris, R.H., and Stauffer, J.F., eds.) pp. 133–147, Burgess Publishing, Minneapolis.
16. Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*, 248–254.
17. Zamora, R., and Hidalgo, F.J. (1995) Influence of Irradiation Time, pH, and Lipid/Amino Acid Ratio on Pyrrole Production During Microwave Heating of a Lysine/(*E*)-4,5-epoxy-(*E*)-2-heptenal Model System, *J. Agric. Food Chem. 43*, 1029–1033.
18. Favier, A.E. (1994) in *Trace Elements and Free Radicals in Oxidative Diseases* (Favier, A.E., Neve, J., and Faure, P., eds.) pp. 57–80, AOCS Press, Champaign.
19. Dillard, C.J., and Tappel, A.L. (1988) in *Cellular Antioxidant Defense Mechanisms* (Chow, C.K., ed.) pp. 103–115, CRC Press, Boca Raton.
20. Gavino, V.C., Dillard, C.J., and Tappel, A.L. (1984) Release of Ethane and Pentane from Rat Tissue Slices: Effect of Vitamin E, Halogenated Hydrocarbons, and Iron Overload, *Arch. Biochem. Biophys. 233*, 741–747.

[Received June 4, 1994, and in revised form March 20, 1995; Revision accepted April 4, 1995]

Plasma and Lipoprotein Lipid Peroxidation in Humans on Sunflower and Rapeseed Oil Diets

Anu M. Turpeinen[a], Georg Alfthan[b], Liisa Valsta[a,1], Eino Hietanen[c,2], Jukka T. Salonen[d], Hanna Schunk[c], Kristiina Nyyssönen[d] and Marja Mutanen[a,*]

[a]Department of Applied Chemistry and Microbiology, Division of Nutrition, SF-00014 University of Helsinki, Finland, [b]Department of Nutrition, National Public Health Institute, SF-00300 Helsinki, Finland, [c]International Agency for Research on Cancer, Lyon, F-69372 Lyon, France and [d]Research Institute of Public Health, University of Kuopio, SF-70211 Kuopio, Finland

ABSTRACT: The effects of natural mixed diets on lipid peroxidation were investigated in humans. In the first study, 59 subjects were fed a rapeseed oil-based diet rich in monounsaturated fatty acids (MUFA) and a sunflower oil-based diet rich in polyunsaturated fatty acids (PUFA) in a cross-over manner for three and a half weeks. The lipid peroxidation products in plasma were determined by measuring conjugated dienes and malondialdehyde (MDA). In a second study, plasma thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides, and the susceptibility of very low density lipoprotein + low-density lipoprotein (LDL) to *in vitro* oxidation were measured from subjects fed similar MUFA and PUFA diets for six week diets. No significant differences in plasma MDA or conjugated diene concentrations were found after the rapeseed oil diet or the sunflower oil diet in Study 1. In the second study, a small but significant decrease ($P < 0.05$) in both lipid hydroperoxides and TBARS was observed in the LDL fraction after the sunflower oil diet. The *in vitro* oxidation gave opposite results, showing increased oxidation after the sunflower oil diet. Despite a high intake of α-tocopherol during the oil periods, no increase in plasma α-tocopherol was noticed in either study. The results suggest that moderate changes in the fatty acid composition in the Western-type diet may be adequate to affect lipoprotein susceptibility to oxidation *in vitro*, but there is considerable disparity with some indices of *in vivo* lipid peroxidation.
Lipids 30, 485–492 (1995).

Increased lipid peroxidation is thought to be involved in a wide variety of clinical diseases and toxicological conditions, including atherosclerosis, diabetes mellitus, cancer, rheumatoid arthritis, and toxicity induced by certain metals, drugs, and pesticides (for review, see Ref. 1). Recently, evidence suggesting lipid peroxidation in human plasma lipoproteins *in vivo* has been presented (2). The composition of cellular membranes and also lipoprotein fatty acids reflects the type of dietary fat (3), and it is frequently assumed that there is a relationship between dietary fat type and tissue susceptibility to lipid peroxidation initiated by oxidative stress. Oxidative stress and subsequent lipid peroxidation *in vivo* may partly depend on the balance between prooxidants (some metals) and antioxidants [vitamin E, ß-carotene, ascorbate, glutathione peroxidase (GHSPx)(4)] in tissues. In animal studies, lipid peroxidation has been shown to differ when the type of dietary fat was changed (5). The amount of lipid peroxidation is largely dependent on the amount of antioxidants, especially vitamin E, in the diet (6–8).

It is not yet established whether dietary fatty acids affect lipid peroxidation in humans consuming an ordinary Western-type diet. In a recent study of 37 men, plasma thiobarbituric acid reactive substances (TBARS) decreased in those on a diet with a higher content of polyunsaturated fatty acids (PUFA) as compared to those on a low-fat diet with lesser amounts of PUFA (9). Generation of conjugated dienes in low-density lipoprotein (LDL) *in vitro*, however, was significantly depressed after consuming a high oleic acid diet as compared to a high linoleic acid diet (10,11). Also, linoleic acid supplementation in humans has led to an increased susceptibility of LDL to *in vitro* oxidation (11,12).

In the present paper, we describe two studies on humans attempting to examine dietary effects on lipid peroxidation. First, we examined the effects of mixed diets of highly different fatty acid composition on plasma lipid peroxidation products and plasma glutathione concentration in healthy men and women in a strictly controlled cross-over design. In the second study, the amounts of TBARS and lipid hydroperoxides

[1]Present address: Department of Nutrition, National Public Health Institute, SF-00300 Helsinki, Finland.

[2]Present address: Department of Clinical Physiology, University of Turku, SF-20520 Turku, Finland.

*To whom correspondence should be addressed at Department of Applied Chemistry and Microbiology, Division of Nutrition, P.O. Box 27, 00014 University of Helsinki, Finland.

Abbreviations: BHT, butylated hydroxytoluene; BuOH, *n*-buthanol; CV, coefficient of variation; en%, percent of energy; GSHPx, glutathione peroxidase; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; PBS, phosphate buffered saline; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

in plasma lipoprotein fractions, and the susceptibility of very low density lipoprotein (VLDL) + LDL to *in vitro*-induced oxidation, were measured after high linoleic or oleic acid diets in 6–7 healthy men.

METHODS

Both studies were approved by the Human Ethical Committee of the Faculty of Forestry and Agriculture at the University of Helsinki (Helsinki, Finland).

Subjects and design of Study 1. Fifty-nine (30 female, 29 male) healthy, normotensive, nonsmoking volunteers entered the first study after giving informed consent. The participants were mainly university students and employees (average age 30 yr, range 18–60 yr) having a normal body mass index of 22.3 ± 2.5 kg/m^2 (mean ± SD). The participants continued their normal activities during the study period.

A detailed description of the study design has been reported (13). The study was performed over three consecutive periods: during the first 14 d, subjects were given a milk fat-based (saturated fat) diet, after which 30 subjects were given a sunflower oil-based (PUFA) diet for 24 d and then a low erucic acid rapeseed oil-based [monounsaturated fatty acids (MUFA)] diet for 24 d. The other 29 subjects had the sunflower oil and the rapeseed oil diets for 24 d each in reverse order. The subjects were aware that they were being given milk fat diet during the first 14 d, but they were unable to determine the order of the oil diets.

The experimental diets contained mixed solid foods customary in the Finnish diet covering about 90% of the energy intake per day. In addition to the food supplied, the subjects were allowed to choose 10% of their daily energy intake from foods free of fat and cholesterol (candies, fruit, beer, etc.). The subjects recorded these foods in their diaries. The diets were planned to have the same energy, total fat, cholesterol, and nutrient content throughout the dietary periods. The differences between the diets were solely in their fatty acid composition (Table 1). The figures are based on chemical analyses of the daily collected duplicate portions in addition to the calculated contribution from the freely chosen foods. The test oils were included in the diets in special margarines, bread, salad dressing, ice cream, buns, pies, cakes, and pancakes. The food for each subject was weighed according to energy intake and adjusted to maintain the actual body weight of the subject throughout the study. The subjects ate lunch at the University premises on working days. Foods for the rest of the day and next morning and for weekends were packed and given to the subjects on Fridays.

Subjects and design of Study 2. In the second study, 13 healthy normotensive, nonsmoking, male volunteers (average age 29 yr, range 21–36 yr) were divided into two groups after giving their informed consent. Seven subjects were fed a high oleic acid rapeseed oil diet, and six subjects a high linoleic acid sunflowerseed oil diet for six weeks in a parallel manner. Approximately 50 g of test oil/day as margarine, salad dressing, cooking oil, oil-enriched bread, cakes, and cookies baked using the test fats were included in normal mixed diets. Fatty fish and fish oil were excluded from the diets. Dietary records were kept by the subjects before the study for three days and for seven days during the six-week study period. The nutrient composition (Table 2) is based on the calculated composition of the diets. The percentage of energy (en%) obtained from MUFA and PUFA in the rapeseed and sunflowerseed oil diets were 18.3 and 9.0, and 12.1 and 15.6, respectively, and the amount of saturated fatty acids was practically the same (12.5 and 12.0 en%). The subjects were asked to continue their normal activities during the study period. Dietary compliance was checked with food records, food disappearance inventory, and plasma fatty acids.

TABLE 1
Mean Daily Intake of Energy and Nutrients According to Duplicate Portion Analysis and Calculated Contribution from Freely Chosen Foods (Study 1)

Nutrient	Baseline diet milk fat	Sunflower oil diet	Rapeseed oil diet
Energy (MJ)	10.1	10.6	10.5
Total fat (en%)[a]	35.8	37.7	37.8
Saturated (en%)	18.9	12.7	12.4
C_{12}–C_{16} (en%)[b]	12.7	8.0	8.1
Stearic acid (en%)	4.7	3.8	3.4
Monounsaturated (en%)	11.0	10.2	16.2
Oleic acid (en%)	9.5	9.6	15.0
Polyunsaturated (en%)	3.7	13.3	7.6
Linoleic acid (18:2n-6)	3.2	12.7	5.5
α-Linolenic acid (18:3n-3)	0.4	0.4	2.1
Carbohydrates (en%)	49.5	48.4	47.7
Protein (en%)	14.9	14.5	15.1
Dietary fiber (en%)	30	30	29
Alcohol (en%)	1.0	1.0	1.1
Cholesterol (mg/day)	354	315	360
P/S[c]	0.2	1.0	0.6
Vitamin A (retinol equivalents/day)[d]	970	590	575
Vitamin E (mg α-Te/day)[d,e]	8.3	27.2	13.7
Selenium (μg/day)[d,e]	111	108	118
Vitamin E/PUFA[e,f]	2.2	2.0	1.8
Vitamin E/MUFA[g]	0.8	2.7	0.8

[a]en%, Percent of energy.
[b]Saturated fatty acids with chain lengths of 12, 14, or 16 carbon atoms.
[c]The ratio between polyunsaturated fatty acids and saturated fatty acids.
[d]Does not include small amounts from freely selected foods.
[e]RDA for vitamin E 8–10 mg α-tocopherol equivalents/day and for selenium 55–70 μg/day.
[f]The ratio between vitamin E and polyunsaturated fatty acid (PUFA) intake.
[g]The ratio between vitamin E and monounsaturated fatty acid (MUFA) intake.

Blood sampling. In the first study, blood was taken from overnight fasted subjects into EDTA (0.1 mL/10 mL; 3.8 mmol/L) by venipuncture once during the last week of each period (baseline milk fat diet, rapeseed oil diet, and sunflower oil diet). The blood samples were centrifuged at 1000 × *g* for 10 min, and plasma was separated within 1 h after venipuncture. Aliquots of the samples with butylated hydroxytoluene (BHT; 1 mmol/L) were quickly frozen to −70°C.

During the second study, blood was taken twice, before

TABLE 2
Mean (SD) Calculated Energy and Nutrient Intake of the Subjects (n = 6 and n = 7) in the Second Study[a]

Nutrient	HABSO (n = 5)	SO (n = 6)	HABRO (n = 7)	RO (n = 7)
Energy (MJ)	13.7 (1.8)	12.3 (0.7)	10.1 (1.4)	11.7 (1.7)
Total fat (en%)	36.6 (6.2)	41.2 (5.0)	36.1 (5.7)	41.5 (3.8)
Saturated (en%)	16.0 (4.0)	12.0 (2.0)	16.2 (3.3)	12.5 (2.4)
Monounsaturated (en%)	14.0 (2.1)	12.1 (1.8)	13.0 (2.6)	18.3 (1.4)
Polyunsaturated (en%)	5.7 (1.0)	15.6 (2.3)	6.0 (1.4)	9.0 (0.6)
Linoleic acid (en%)	4.7 (0.9)	14.9 (2.3)	4.8 (1.6)	6.4 (0.4)
α-Linolenic acid (en%)	0.6 (0.1)	0.4 (0.1)	0.4 (0.1)	2.4 (0.2)
Carbohydrates (en%)	50.0 (8.5)	46.3 (4.6)	46.0 (6.4)	41.2 (3.8)
Protein (en%)	13.8 (1.9)	11.3 (1.3)	15.5 (2.1)	12.6 (0.5)
Alcohol (en%)	0.9 (1.9)	1.2 (1.5)	2.4 (2.0)	4.0 (3.0)
Cholesterol (mg/day)	508 (137)	287 (65)	410 (205)	327 (135)
P/S ratio[b]	0.36 (0.13)	1.30 (0.24)	0.37 (0.10)	0.72 (0.14)
Vitamin A (retinol equivalents/day)	2344 (1643)	3328 (2344)	2928 (1674)	3335 (2665)
Vitamin E (mg α-Te/day)	14.9 (2.6)	49.6 (6.9)	12.2 (6.0)	21.5 (3.7)
Vitamin E/PUFA	2.6	3.2	2.0	2.4
Vitamin E/MUFA	1.1	4.1	0.9	1.2

[a]HABSO, habitual diet before sunflower oil diet; SO, sunflower oil diet; HABRO, habitual diet before rapeseed oil diet; RO, rapeseed oil diet; en%, percent of energy. Other abbreviations as in Table 1.
[b]The ratio between polyunsaturated fatty acids and saturated fatty acids.

and at the end of the six-week oil supplementation, into EDTA (0.1 mL/10 mL; 3.8 mmol/L). Plasma was separated as described previously, and the samples were kept at 4°C until the lipoprotein fractions were separated. Separation of different lipoprotein fractions was carried out within 48 h of the venipuncture.

Measurement of plasma malondialdehyde (MDA). MDA was analyzed spectrophotometrically in protein-free plasma and by high-performance liquid chromatography (HPLC) with fluorometric detection in plasma lipid fractions.

Spectrophotometric detection of MDA. The spectrophotometric detection of MDA was based on determination of MDA as a thiobarbituric acid (TBA)–MDA adduct after protein precipitation (14). Two mL of TBA-solution (0.67% glacial acetic acid/water, 50:50, vol/vol), 0.28 mL of BHT (1.0 mM final concentration), and 0.07 mL $FeCl_3$ (0.25 mM final concentration) were added to clear supernatant (0.5 mL), and the samples were heated for 60 min at 100°C, cooled, and centrifuged at 25°C 12000 × *g* for 15 min. MDA was detected at 532 nm. MDA-*bis*-(diethylacetal) standards (from 0.49 to 3.95 μmol/L) were prepared daily.

HPLC–fluorometric detection of MDA. Plasma samples with lipid extraction and HPLC separation of the TBA–MDA adduct were analyzed with a slight modification from the method of Wade and van Rij (15). MDA-*bis*-(diethylacetal) (Merck, Schuchardt, Germany) was used as a standard instead of 1,1,3,3-tetraethoxypropane. The standards (0.49, 0.99, 1.98, and 2.97 μmol MDA/L) were prepared daily. All the samples from each subject were analyzed on the same day with an internal standard (serum pool) to eliminate within-series variation.

After hexane/isopropanol (2:3, vol/vol; 5.0 mL) extraction, plasma samples were centrifuged (200 × *g* for 15 min), and 2.5 mL of the supernatant was separated and dried under nitrogen using a 30°C waterbath. Triton X-100 (10%; 0.1 mL), acetic acid (50%; 0.5 mL), TBA (0.67% in glacial acetic acid/water, 50:50, 1.0 mL), BHT (0.1 M; 50 μL), and $FeCl_3$ (0.01 M; 50 μL) were added in this order to the dried samples. The final concentrations of $FeCl_3$ and BHT were 0.25 and 1.0 mM, respectively. $FeCl_3$ was used to stimulate the degradation of all lipid hydroperoxides to MDA (15). BHT was added just before the iron to prevent the peroxidation of intact fatty acids. The samples were heated at 100°C for 60 min, cooled and, after addition of 2 mL of 2% NaCl, the TBA–MDA adduct was extracted to 1.5 mL of *n*-buthanol (BuOH). Samples were centrifuged (5 min 200 × *g*), and fluorometric detection of TBA–MDA complex was made on the BuOH supernatant (excitation 515 nm/emission 553 nm). The chromatographic column used was a self-made RP18 Lichrosorb 9333 (150 mm; 5 μm particle diameter), and the mobile phase was MeOH/acetic acid 50 mM/L (80:20), pH 5 (flow rate 0, 75 mL/min).

Measurement of conjugated dienes. Conjugated dienes were measured spectrophotometrically from plasma. After lipid extraction with hexane/isopropanol (3:4, vol/vol), the samples were centrifuged for 10 min at 200 × *g* and the absorbance was measured at 235 nm. The final concentration of conjugated dienes was calculated using the molar absorbance for conjugated dienes (27,000 $M^{-1}cm^{-1}$) (8).

Total GSH measurement. GSH was measured using the method of Saville (16) and modified by Heinonen and Vainio (17). Briefly, plasma (0.1 mL) was mixed with 0.2 mL of 10 mM *bis*(3-carboxy-4-nitrophenyl)disulfide and centrifuged for 6 min at 2000 × *g*. Oxidized GSSG and reduced NADPH were added to the supernatant, and the absorbance was measured at 412 nm.

Separation of lipoprotein fractions. Lipoprotein fractions were separated in the second study by gel permeation chro-

matography using an application (18) from the general scheme (19) with one ultracentrifugation step (50.4 Ti rotor, Beckman XL-90; Beckman Instruments, Palo Alto, CA) and gel chromatography on modified agarose columns. The total lipoproteins were prepared by adding solid potassium bromide to 6 mL of plasma to obtain the required density of 1.22 g/mL (20) and centrifuging the sample in Beckman polyallomer tubes for 23 h at 218,000 × *g* and 10°C. The removed lipoprotein fraction was concentrated at +4°C (Centricon miniconcentrators; Amicon, Danvers, MA), with a cut-off at 10^5 daltons. The filtered samples (0.2 μm) were injected into the gel chromatographic column. A fast protein liquid chromatographic system at +10°C was used. Samples were introduced *via* a P-1 peristaltic pump and an MV-7 motor valve (Pharmacia, Uppsala, Sweden) with a 500 μL loop. Two Superose 6 (Pharmacia) columns were used in series for the separation of the lipoprotein fractions. The mobile phase consisted of sodium phosphate buffer with 1 mM EDTA and 0.02% NaN_3 (pH 7.2). The elution of lipoproteins was monitored at 280 nm.

The TBARS in lipoprotein fractions were measured fluorometrically (21) directly on 1 mL of sample mixed with 600 μL of 40% trichloroacetic acid and 1 mL of 0.8% TBA without any sample pretreatment. The method was calibrated using 1,1,3,3-tetraethoxypropane as a standard. The protein content of the lipoprotein fractions was measured as described previously (18).

Tri-iodide test for the determination of lipid hydroperoxides. Lipoprotein fraction lipid hydroperoxides were measured spectrophotometrically at 365 nm (22,23). Two hundred μL of 3% acetic acid and 50 μL of 1 mmol/L I_2 solution were added to a 300 μL aliquot of the lipoprotein sample (adjusted to 0.025 mg protein/mL), and the mixture was incubated for 10 min at room temperature. A color reagent (500 μL, CHOD-iodide, Cat. no. 14106; Merck, Darmstadt, Germany) was added, and after 1 h of incubation at room temperature the reaction was stopped by adding 200 μL of 7% Nonidet P40 (BDH, Poole, England). Hydrogen peroxide (50 μmol/L) was used as a standard in every run, together with samples. A sample blank was prepared for every sample by adding Nonidet P40 to the reaction mixture before the color reagent.

Copper-induced oxidation of the VLDL + LDL fraction. Combined VLDL and LDL were isolated by ultracentrifugation in a Beckman XL-90 ultracentrifuge for 23 h at 4°C in a potassium bromide gradient containing 0.2 mmol/L of EDTA. The top layer from the tube (<1.063 g/mL density) was collected with a pipette and assayed immediately. EDTA and other salts were removed from the VLDL + LDL fraction by using PD-10 columns (Pharmacia). VLDL + LDL was diluted to 1.5 mL with phosphate-buffered saline (PBS; 0.16 M sodium chloride, 0.01 M sodium phosphate, pH 7.4), poured into the column and eluted with PBS. The first 0.8 mL was discarded, and the next 1.6 mL was collected. VLDL + LDL was diluted with oxygen-saturated PBS to a protein concentration of 0.05 mg/mL. The reaction was started by adding 33.5 μL of 0.1 mM $CuCl_2$ to 2 mL of diluted VLDL + LDL fraction and followed at 37°C by a temperature-controlled spectrophotometer with an enzyme kinetic data system (Hitachi U-2000; Hitachi, Tokyo, Japan) for 3 h. The measurement wavelength was 234 nm. Maximum oxidation velocity (V_{max}), the time from the start of the reaction to the steepest slope (lag time), the maximum absorbance of oxidation, and the time for maximum absorbance was computed from the reaction curve.

Statistical analysis. Statistical analysis of the data from the spectrophotometric TBA method with protein precipitation (Study 1) was based on the model of univariate analysis of variance taking repeated measurements into account (2 × 2 cross-over trial)(24,25). The factors studied were carry-over effect (any possible carry-over effect from one oil diet to another), period effect, and treatment effect. No evidence was found to suggest a carry-over effect between the diet treatment sequences. The data from the HPLC–fluorometric TBA method and from the conjugated dienes was analyzed using Student's *t*-test for paired data, comparing the different dietary periods. The effects of age and sex were also studied using Student's *t*-test for paired data and one-tailed analysis of variance. The results of *in vitro*-induced oxidation, TBARS, and lipid hydroperoxides were tested with the unpaired Wilcoxon Signed Rank test (Study 2). A carry-over effect between the diet treatment sequences was found when analyzing the GSH data, and the data for the two groups could not be combined.

RESULTS

The first study was strictly controlled, and therefore it was possible to obtain a great difference in the fatty acid composition of the diets in three periods (Table 1). In the baseline milk fat period, nearly 19 en% was from saturated fatty acids and only 3.2 en% from linoleic acid. During the two oil periods, less than 13 en% originated from the saturates. During the sunflower oil period, 13.3 en% was covered by polyunsaturates and 10.2 en% by monounsaturates. The respective figures in the rapeseed oil period were 7.6 and 16.2 en%. The analyzed nutritional values from duplicate portion analysis came very close to the calculated values (95–98% for total fat in the three diets, 92–95% for saturated fat, 95–98% for MUFA, 95–99% for PUFA, and 73–81% for cholesterol). Dietary compliance was evaluated by monitoring the ratio of oleic (18:1n-9) to linoleic (18:2n-6) acid in the plasma phospholipids. The mean ratio was 0.49 ± 0.07 at the end of the saturated baseline diet and was reduced to 0.27 ± 0.04 during the sunflower oil diet ($P < 0.001$) and increased to 0.56 ± 0.07 during the rapeseed oil diet ($P < 0.01$). No significant differences between the oil periods were found in the plasma concentrations of either MDA or conjugated dienes. After the monounsaturated rapeseed oil period, the plasma level of spectrophotometrically detected MDA was lower than the amount of MDA in the baseline milk fat period ($P < 0.01$, Table 3). Vitamin E intake was very high (27.2 mg/d according to duplicate portion analysis) when the subjects were on

TABLE 3
Mean (SD) Plasma Lipid Peroxidation [malondialdehyde (MDA) and conjugated dienes] and GSH Levels in the First Study

	Baseline diet milk fat	Sunflower oil diet	Rapeseed oil diet
MDA μmol/L, method 1[a]	1.13 (0.41) n = 19	1.11 (0.39) n = 28	1.11 (0.30) n = 28
MDA μmol/L, method 2[a]	7.52 (3.66) n = 53	7.25 (2.94) n = 53	6.29 (3.05) n = 57[b]
Conjugated dienes μmol/L	5.21 (1.69) n = 17	6.03 (2.70) n = 12	4.79 (2.43) n = 24
Glutathione μmol/L, group 1	1.49 (0.23) n = 30	1.49 (0.26) n = 30	
Glutathione μmol/L, group 2	1.79 (0.32) n = 28		1.65 (0.34) n = 28[c]

[a]Method 1 was high-performance liquid chromatography–fluorometric and method 2 spectrophotometric measurement of MDA in plasma samples.
[b]Significant difference between baseline diet and rapeseed oil diet: $P = 0.007$.
[c]Significant difference between baseline diet and rapeseed oil diet: $P = 0.01$.

the sunflower oil diet and the intake was also well above recommendations during the rapeseed oil diet (Table 1). Plasma α-tocopherol increased from 4.8 to 6.35 mg/L during the rapeseed oil diet, but no significant changes were observed during the sunflower oil diet (after milk fat diet vs. after sunflower oil diet; 6.4 ± 1.5 vs. 6.9 ± 1.6 mg/L). When taking the initial concentrations into account, the difference between the diets was not significant. The ratio of plasma α-tocopherol/LDL was also calculated, and a significant increase was observed after both diets (milk fat vs. PUFA diet; 4.7 ± 1.0 vs. 6.0 ± 2.0, $P < 0.05$; milk fat vs. MUFA diet; 4.8 ± 1.5 vs. 7.0 ± 1.6, $P < 0.001$).

GSH results could be analyzed only for the first dietary treatment period (half of the subjects), because of a carry-over effect found (Table 3). Sunflower oil diet (1.49 ± 0.26 μmol/L) had no effect on GSH values as compared to baseline milk fat diet (1.49 ± 0.23 μmol/L, group 1), whereas the initially higher baseline values (1.79 ± 0.32 μmol/L) of group 2 were significantly decreased by the rapeseed oil diet (1.65 ± 0.34 μmol/L, $P < 0.05$).

Considerable differences between the intakes of MUFA and PUFA were achieved in the second study (Table 2). Several methods were used to check the compliance of the subjects. The nutrient intake from food records showed at group level that the experimental fats and foods had been used. In accordance with the food record data, the food disappearance inventory reflected an 85% compliance to the test food intake on average. Also, the proportion of α-linolenic acid in plasma cholesterol esters increased from 0.9 to 1.2% by the rapeseed oil diet and decreased from 0.9 to 0.4% by the sunflower oil diet ($P < 0.001$ at the end of the test periods), all showing a good compliance to the diets.

The amounts of TBARS and lipid hydroperoxides were measured in VLDL, intermediate-density lipoprotein, LDL, and high-density lipoprotein fractions. Small but significant differences between the treatments ($P < 0.05$) were found

TABLE 4
Mean (SD) Thiobarbituric Acid Reactive Substances (TBARS) and Lipid Hydroperoxide Contents of the Main Plasma Lipoprotein Fractions and *in vitro* Oxidation Parameters of VLDL + LDL Before and After the Six-Week Sunflower and Rapeseed Oil Diet Periods (Study 2)

Lipoprotein fraction[a]	Sunflower oil diet			Rapeseed oil diet			Significance between groups[b]
	Before	After	Change	Before	After	Change	
TBARS (μmol/g)[c]							
VLDL	0.58 (0.21)	0.49 (0.39)	−0.09 (0.40)	0.36 (0.12)	0.23 (0.10)	−0.12 (0.11)	ns.[a]
IDL	0.27 (0.14)	0.30 (0.21)	0.03 (0.25)	0.25 (0.09)	0.30 (0.30)	0.05 (0.32)	ns.
LDL	0.39 (0.44)	0.14 (0.06)	−0.25 (0.46)	0.20 (0.08)	0.24 (0.08)	0.04 (0.10)	0.046
HDL	0.11 (0.11)	0.06 (0.03)	−0.05 (0.11)	0.14 (0.22)	0.05 (0.02	−0.09 (0.21)	ns.
Lipid hydroperoxides (μmol/g)[c]							
VLDL	2388 (138)	2238 (151)	−150 (152)	2417 (154)	2253 (118)	−164 (73)	ns.
IDL	2372 (197)	2192 (172)	−181 (121)	2446 (237)	2421 (394)	−26 (382)	ns.
LDL	2606 (165)	2194 (202)	−412 (291)	2554 (233)	2423 (220)	−131 (170)	0.046
HDL	2615 (264)	2275 (185)	−341 (139)	2617 (182)	2502 (295)	−155 (246)	ns.
In vitro oxidation of VLDL + LDL							
Lag time (min)	37.6 (3.8)	30.0 (0.0)	−7.5 (2.7)	38.6 (3.8)	41.4 (4.8)	2.9 (6.4)	0.008
V_{max} (mabs/min)	11.6 (0.9)	13.4 (1.7)	1.8 (1.6)	10.4 (0.8)	10.1 (1.2)	−0.3 (1.1)	0.032
Max oxidation (abs)	0.53 (0.06)	0.55 (0.08)	0.02 (0.08)	0.49 (0.03)	0.48 (0.09)	−0.004 (0.074)	ns.
Time to max oxidation (min)	78.3 (4.1)	69.2 (3.8)	−9.2 (3.8)	81.4 (4.8)	85.0 (5.8)	3.6 (8.0)	0.011

[a]VLDL, very low density lipoprotein; IDL, intermediate-density lipoprotein; HDL, high-density lipoprotein; ns., nonsignificant. V_{max}, maximum oxidation velocity.
[b]Wilcoxon Rank Sum test for paired samples.
[c]Expressed per g of protein.

only in the LDL fraction (Table 4). The change in the production of lipid hydroperoxides was −412 ± 291 μmol/g for the sunflower oil period and −131 ± 170 μmol/g for the rapeseed oil period. The respective figures for plasma TBARS were −0.25 ± 0.46 μmol/g and 0.04 ± 0.10 μmol/g. The *in vitro* Cu^{2+}-induced oxidation of VLDL + LDL gave very different results. The lag time and time to maximum oxidation decreased during the sunflower oil diet and increased during the rapeseed oil diet. The differences for the changes between diets were significant ($P < 0.01$ for lag time and $P < 0.05$ for time to maximum oxidation). The oxidation velocity increased during the sunflower oil diet and slightly decreased during the rapeseed oil diet ($P = 0.032$ for significance between diets). The maximum absorbance in Cu^{2+}-induced oxidation did not change significantly during either diet. There were no significant differences in plasma concentrations of α-tocopherol between the polyunsaturated (baseline vs. intervention; 8.58 ± 3.24 vs. 7.89 ± 1.00 mg/L) and monounsaturated (8.79 ± 1.33 vs. 7.67 ± 1.01 mg/L) study groups. No changes were observed in the plasma α-tocopherol/LDL ratio after the MUFA diet (baseline vs. intervention; 8.7 ± 0.8 vs. 10.7 ± 4.0, $P = 0.230$) or the PUFA diet (baseline vs. intervention; 6.8 ± 2.5 vs. 7.1 ± 1.7, $P = 0.831$).

DISCUSSION

The purpose of our controlled diet study was to evaluate the effects of PUFA and MUFA diets on plasma lipid peroxidation levels, when consumed in amounts practical for mixed diets. The very different fatty acid composition of the oil diets had, however, no marked effects on the indicators of lipid peroxidation used in the first study. In the rapeseed oil diet group, however, less MDA was present when measured by the spectrophotometric method. The aim of our second study was to find out if these types of diets have any special effect on the *in vitro*-induced oxidation of LDL or on the levels of TBARS or lipid hydroperoxides in lipoprotein fractions. The results of lipoprotein oxidation resistance, TBARS, and lipid hydroperoxides are rather controversial. TBARS and lipid hydroperoxides measured by the tri-iodide test from lipoprotein fractions indicate that dietary PUFA do not increase the content of oxidation products in plasma lipoproteins. On the contrary, the results from Cu^{2+}-induced oxidation resulted in three of four parameters that the PUFA diet significantly decreased the oxidation resistance of lipoproteins.

There are only two strictly controlled human studies where normal foods have been used as components of experimental diets. In a study from Israel, a PUFA diet induced a significantly higher tendency toward plasma lipid peroxidation (as ascertained by more TBARS formed) compared with a MUFA diet (26). However, moderate amounts of PUFA (7.9 en%) decreased plasma TBARS as compared to a low-fat diet with a low content of polyunsaturates (3.2 en%) in free-living Finnish men (9). The TBARS results of our second experiment are in line with the latter results and also with those obtained from healthy subjects showing a negative correlation between serum MDA concentration and the lipoprotein linoleic acid content (27). It is known, however, that plasma MDA is largely generated by platelet aggregation during *ex vivo* clot formation, and so is unlikely to reflect lipid peroxidation *in vivo* (28).

In several recent studies, the effects of different fatty acids on the *in vitro* oxidation of LDL have been studied. Either liquid diets or oil supplements have been used as sources of the fatty acids under investigation, so these diets cannot be referred to as diets of normal, healthy, free-living humans. No difference in the susceptibility to oxidation has been found between fish oil (1 g n-3 fatty acids/day) and corn oil supplemented diets (29) or after a diet rich in *trans* fatty acids (7 en%)(30). Our results from Study 2 are in line with two other studies where oleate-rich diets (28.7 and 16.8 en% monounsaturated as compared to 12.1–18.3 en% in our study) have decreased and linoleate-rich diets (50.9 and 12.2 en% of PUFA as compared to 9.0–15.6 en% in our study) have increased the *in vitro* oxidability of LDL measured as in the production of conjugated dienes (3,11).

It is widely assumed that LDL particles rich in PUFA should be more readily oxidized than those with less unsaturated fatty acids. *In vivo* antioxidant availability may be an even more important factor than substrate in the potential oxidation of PUFA (11). As shown in Table 1, the intake of vitamin E increased considerably with the intake of PUFA. The ratio of vitamin E/PUFA intake, however, was actually fairly constant within diets (1.8–2.2 in Study 1 and 2.0–3.2 in Study 2). No change in plasma α-tocopherol content was noticed after the diets in either study, indicating higher endogenous consumption of vitamin E during the high vitamin E sunflower oil diets. Some animal (7,11) and human (31,32) experiments using high PUFA fish oils with different amounts of vitamin E support this assumption. In line with this, the urinary excretion of MDA decreased when a high level of antioxidants (vitamin E and C, BHT) was fed to rats (33). A significant increase in the ratio α-tocopherol/LDL was observed after both diets in Study 1, but not in Study 2. This may be partly explained by a higher interindividual variation in dietary and plasma α-tocopherol in Study 2, due to a less strict study design. The decrease in LDL cholesterol was also higher in Study 1 (on average 22%) than in Study 2 (on average 18%).

As the substrate for GSHPx, reduced GSH effectively protects tissues and cellular constituents from the damaging effects of hydroperoxides formed during normal metabolism. Decreased plasma and tissue GSH concentrations or increased GSHPx concentrations have been associated with cell damage (34), depressed immunity (35), lipid peroxidation (36), and the progression of aging (37). Although the decrease in plasma GSH following the monounsaturated rapeseed oil diet in our first experiment was quite small, it may indicate increased GSH consumption and elevated oxidative stress. It is worth emphasizing that the initial GSH level was clearly higher in group 2 (having rapeseed oil diet) when compared to group 1 (having sunflower oil in their diet), and in spite of a significant decrease during the rapeseed oil period the

plasma GSH level did not reach the level found in the sunflower oil group.

The other purpose of our studies was to compare different methods for measuring lipid peroxidation. It seems that the amount of lipid peroxidation in human blood plasma and lipoproteins varies greatly depending on the method used (Table 3). The highly different results obtained with different methods can partly be attributed to the fact that lipid peroxidation was measured in different phases of the chain reaction. Conjugated dienes are formed during the rearrangement of double bonds in fatty acids after hydrogen atom abstraction by a free radical. Conjugated dienes are therefore formed when peroxidation begins. However, only fatty acids with three or more double bonds produce MDA as an end product. The sensitivity of these methods is obviously not the reason for not detecting any differences in lipid peroxidation, as the detection limit of these methods is picomoles per liter, e.g., 8.3 pmol for the fluorometric method (38).

Plasma MDA (1.11–1.13 μmol/L) was easily detected with the HPLC–fluorometric method as it was the only compound at the wavelengths used. The results were also quite consistent with the latest results from other studies (14,38). Interestingly, in this study the HPLC method gave slightly, but not significantly, lower mean plasma MDA concentrations for men than for women (data not shown), contrary to results obtained by Knight *et al.* (39). This may be partly explained by slightly, but not significantly lower serum total and LDL cholesterol of men in this study. Knight *et al.* (39) also observed that the plasma concentration of MDA rose with age. In this study, the women were slightly older than the men (32.8 ± 14.1 vs. 27.9 ± 9.0 yr). There was no correlation between age and plasma MDA in different dietary periods (r = –0.096–0.253). A weak correlation (r = 0.275) between age and the concentration of conjugated dienes in human serum has been found by Fink *et al.* (40).

The protein-free plasma samples with spectrophotometric detection gave 6–7 times higher MDA values (6.3–7.9 μmol/L) than those obtained with fluorometric detection. The reproducibility of the spectrophotometric method, which was quite poor [coefficient of variation (CV) was 18% and day-to-day variation 8.7%], may partly explain the unexpected result obtained between habitual milk fat diet and rapeseed oil diet obtained with this method (Table 3). Various other compounds react with TBA and/or MDA, and the varying concentrations of these compounds or varying degrees of *ex vivo* artifact were obviously the reason for the variance found in the results. The Fe^{3+} concentration of samples markedly affects the results and the amount of MDA in samples by stimulating the peroxidation and degradation of lipids and lipid hydroperoxides. Fe^{3+} is normally bound in the body, but conditions in which the bound Fe^{3+} is released may, however, occur in healthy humans as a result of normal metabolism in exercising muscles or hypoxic tissues when the pH falls under 6 (1). The Fe^{3+} concentration may therefore vary from subject to subject. This was, however, taken into account by adding a sufficiently high amount of Fe^{3+} (14).

The concentration of conjugated dienes, 4.79–6.03 μmol/L, fell within the results obtained with the two other methods. The precision of this method was quite good, CV being only 2.1%. The measurement of conjugated dienes is a simple and quick method and suitable for routine analysis. However, methods for the determination of conjugated dienes do not discriminate between those arising from lipid peroxidation and those from dietary sources, e.g., cow's milk, lard, and pork fat (41). Therefore, dietary conjugated dienes may raise plasma concentrations during the milk fat diet. Despite this, a tendency for the highest concentration of conjugated dienes was found after the sunflower oil period, which is in accordance with the theory that diene conjugation in humans is associated mainly with the metabolism of linoleic acid (42). Plasma conjugated dienes have been measured in only a few human studies. Fink *et al.* (40) studied healthy controls and patients with various diseases. Using an HPLC-based method for determining the main diene conjugated derivative of linoleic acid [18:2(9,11)], the molar concentration in healthy humans was 18.3 ± 5.8 μmol/L in men and 23.6 ± 6.9 μmol/L in women. The concentration for healthy subjects is, however, 3–4 times higher than we observed, possibly due to methodological differences or higher mean age of subjects.

The day-to-day CV for the determination of TBARS from lipoprotein fractions was 7.8% (n = 6). Although the CV for lipid hydroperoxides by tri-iodide test was worse (15.6%), both methods gave the same significance between sunflower oil and rapeseed oil groups (Table 4). The lipoprotein fractions for the analysis of TBARS, lipid hydroperoxides, and *in vitro* oxidation were separated within 48 h after sampling, and the fractions were stored frozen. Both samples (before and after diet) of a subject were analyzed in the same batch after the study in order to reduce the variation of the results.

In conclusion, the discrepancy between the results with different methods in Study 2 (TBARS, lipid hydroperoxides, *in vitro* oxidation) shows very clearly the methodological difficulties of lipid peroxidation measurements and their extrapolation to the *in vivo* situation. The measurement of plasma TBARS has been widely criticized and partly replaced by the method of *in vitro* oxidation of LDL, but information obtained by the latter method is of uncertain relevance to atherogenesis. Our results using the latter method agree well with those from other supplementation studies with similar fatty acid contents. These results also suggest that moderate changes in mixed diets may be adequate to affect the susceptibility of lipoproteins to oxidation. Further work will be needed, however, to establish whether any relationship exists between a tendency to chemical oxidation *in vitro* and disease processes associated with *in vivo* lipid peroxidation.

ACKNOWLEDGMENTS

We thank Marina Heinonen, D.Sc., for analyzing diet samples for vitamin A and E. The work was supported by grants from the Academy of Finland, the Finnish Cultural Foundation, the Food Research

Foundation, the Sigrid Juselius Foundation, the Ministry of Agriculture and Forestry, and the Yrjö Jahnsson Foundation. The test fats and some of the foods were kindly supplied by the Finnish food industry.

REFERENCES

1. Halliwell, B. (1987) *Ann. Intern. Med. 107*, 526–545.
2. Palinski, W., Rosenfeld, M.E., Ylä-Herttuala, S., Curtner, G.C., Socher, S.S., Butler, S.W., Parthasarathy, S., Carew, T.E., Steinberg, D., and Witztum, J.L. (1989) *Proc. Natl. Acad. Sci. USA 86*, 1372–1376.
3. Angelico, F., Arca, M., Calvieri, A., Cantafora, A., Guccione, P., Monini, P., Montali, A., and Ricci, P. (1983) *Prev. Med. 12*, 124-127.
4. Duthie, G.G., Wahle, K.W.J., and James, W.P.T. (1989) *Nutr. Res. Rev. 2*, 51–62.
5. Hietanen, E., Bartsch, H., Bereziet, J.-C., Ahotupa, M., Camus, A.-M., Cabral, J.R.P., and Laitinen, M. (1990) *Int. J.Cancer 46*, 640–647.
6. Nalbone, G., Leonardi, J., Termine, E., Portugal, H., Lechene, P., Pauli, A.-M., and Lafont, H. (1989) *Lipids 24*, 179–186.
7. Leibovitz, B.E., Hu, M.-L., and Tappel, A.L. (1990) *Lipids 25*, 125–129.
8. Hu, M.-L., Frankel, E.N., Leibovitz, B.E., and Tappel, A.L. (1989) *J. Nutr. 119*, 1574–1582.
9. Sarkkinen, E.S., Uusitupa, M.I.J., Nyyssönen, K., Parviainen, M., Penttilä, I., Salonen, J.T. (1993) *Eur. J. Clin. Nutr. 47*, 623–630.
10. Reaven, P., Parthasarathy, S., Grasse, B.J., Miller, E., Almazan, F., Mattson, F.H., Khoo, J.C., Steinberg, D., and Witztum, J.L. (1991) *Am. J. Clin. Nutr. 54*, 701–706.
11. Abbey, M., Belling, G.B., Noakes, M., Hirata, F., and Nestel, P.J. (1993) *Am. J. Clin. Nutr. 57*, 391–398.
12. Berry, E.M., Eisenberg, S., Haratz, D., Friedlander, Y., Norman, Y., Kaufman, N.A., and Stein, Y. (1991) *Am. J. Clin. Nutr. 53*, 899–907.
13. Valsta, L.M., Jauhiainen, M., Aro, A., Katan, M.B., and Mutanen, M. (1992) *Arteriosclerosis Thromb. 12*, 50–57.
14. Buege, J., and Aust, S. (1978) *Methods Enzymol. 52*, 302–310.
15. Wade, C., and van Rij, A. (1988) *Life Sciences 43*, 1085–1093.
16. Saville, B. (1958) *Analyst 83*, 670–672.
17. Heinonen, T., and Vainio, H. (1981) *Int. J. Drug Metal Pharmacokin. 6*, 275–280.
18. Nyyssönen, K., and Salonen, J.T. (1991) *J. Chromatogr. 570*, 382–389.
19. Rudel, L.L., Lee, J.A., Morris, M.D., and Felts, J.M. (1974) *Biochem. J. 139*, 89–95.
20. Rudel, L.L., Marzetta, C.A., and Johnson, F.L. (1986) *Methods Enzymol. 129*, 45–57.
21. Yagi, K. (1976) *Biochem. Med. 15*, 212–216.
22. El-Saadani, M., Esterbauer, H., El-Sayed, M., Goher, M., Nassar, A.Y., and Jürgens, G. (1989) *J. Lipid Res. 30*, 627–630.
23. Babiy, A.V., Gebicki, J.M., and Sullivan, D.R. (1990) *Atherosclerosis 81*, 175–182.
24. Dixon, W.J., Brown, M.B., Engelman, L., Hill, M.A., and Jennrich, R.I. (1988) *BMDP Statistical Software Manual*, 2nd edn., pp. 1081–1114, University of California Press, Berkley.
25. Jones, B., and Kenward, M.G. (1989) *Design and Analysis of Cross-over Trials*, 1st edn., pp. 88, Chapman and Hall, London.
26. Berry, E.M., Eisenberg, S., Haratz, D., Frielander, Y., Norman, Y., Kaufmann, N.A., and Stein, Y. (1991) *Am. J. Clin. Nutr. 53*, 899–907.
27. Öhrvall, M., Tengblad, S., Ekstrand, B., and Vessby, B. (1993) Abstract, 62th EAS Congress, Jerusalem, Israel, 5–9.9.
28. FitzGerald, G.A., and Patrono, C. (1983) *Circulation 67*, 1174–1177.
29. Nenseter, M.S., Rustan, A.C., Lund-Katz, S., Soyland, E., Maelandsmo, G., Phillips, M.C., and Drevon, C.A. (1992) *Arteriosclerosis Thromb. 12*, 369–379.
30. Nestel, P., Noakes, M., Belling, B., McArthur, R., Clifton, P., Janus, E., and Abbey, M. (1992) *J. Lipid Res. 33*, 1029–1036.
31. Haglund, O., Luostarinen, R., Wallin, R., Wibell, L., and Saldeen, T. (1991) *J. Nutr. 121*, 165–169.
32. Meydani, M., Natiello, F., Goldin, B., Free, N., Woods, M., Schaefer, E., Blumberg, J.B., and Gorbach, S.L. (1991) *J. Nutr. 121*, 484–491.
33. Dhanakoti, S.N., and Draper, H.H. (1987) *Lipids 22*, 643–646.
34. Jain, A., Martensson, J., Stole, E., Auld, P.A.M., and Meister, A. (1991) *Proc. Natl. Acad. Sci. USA 88*, 1913–1917.
35. Franklin, R.A., Li, Y.M., Arkins, S., and Kelley, K.W. (1990) *J. Nutr. 120*, 1710–1717.
36. Li, Y., and Nan, B.S. (1989) *Chin. Med. J. 102*, 670–671.
37. Stohs, S.J., Lawson, T., and Al-Turk, W.A. (1984) *Gen. Pharmacol. 15*, 267–270.
38. Tatum, V., Changchit, C., and Chow, C. (1990) *Lipids 25*, 226–229.
39. Knight, J.A., Pieper, R., and McClellan, L. (1988) *Clin. Chem. 34*, 2433–2438.
40. Fink, R., Clemens, M.R., Marjot, D.H., Patsalos, P., Cawood, P., Norden, A.G., Iversen, S.A., and Dormandy, T.L. (1985) *Lancet II*, 291–294.
41. Thompson, S., and Smith, M.T. (1985) *Chem.-Biol. Interact. 55*, 357–366.
42. Iversen, S.A., Cawood, P., Madigan, M.J., Lawson, A.M., and Dormandy, T.L. (1984) *FEBS 171*, 320–324

[Received February 22, 1994, and in revised form February 25, 1995; Revision accepted March 31, 1995]

9-*cis* β-Carotene in Human Plasma and Blood Cells After Ingestion of β-Carotene

Hiroshi Tamai*, Takao Morinobu, Takuji Murata, Mitsuhiro Manago and Makoto Mino

Department of Pediatrics, Osaka Medical College, Takatsuki, Osaka 569, Japan

ABSTRACT: For 44 wk, thirty male volunteers were given daily either 60 mg of synthesized all-*trans* β-carotene, a naturally-occurring β-carotene derived from *Dunaliella bardawil*, or a placebo. Basal levels of 9-*cis* β-carotene in plasma, platelets, and mononuclear cells were 10, 20, and 25% of those of the all-*trans* form, respectively. The plasma levels reached a maximum after two weeks of administration and plateaued thereafter in the subjects who took the β-carotene preparations. The all-*trans* β-carotene level in the subjects given the synthesized all-*trans* form was almost twice that for the *Dunaliella* preparation. The plasma 9-*cis* level was found to be higher in the all-*trans* β-carotene group than in the *Dunaliella* group, despite no intake of the 9-*cis* form in the all-*trans* group and the higher intake of the 9-*cis* form in the *Dunaliella* group. This finding suggests that isomerization of the all-*trans* form to the 9-*cis* form may occur in the body either during or after absorption.
Lipids 30, 493–498 (1995).

Naturally-occurring carotenoids have recently attracted much attention, due to extensive epidemiological evidence and intervention studies which indicate their potential usefulness in cancer prevention (1,2). However, little is known about the metabolism of β-carotene, the most potent precursor of vitamin A, contained in significant amounts in fruit and vegetables. The halotolerant alga *Dunaliella bardawil* and the closely related species *D. salina* have the unique capacity to accumulate naturally-occurring β-carotenes intracellularly, especially all-*trans* β-carotene and 9-*cis* β-carotene. With respect to bioavailability of the naturally-occurring β-carotenes, a few investigations have shown that all-*trans* β-carotene is predominant in human plasma (3–5), whereas only a small amount of the geometrical isomers of all-*trans* β-carotene is found, even if a large amount of β-carotene isomer derived from alga has been taken orally (6-8). Little is known about the absorption of 9-*cis* β-carotene, which is the second most predominant form contained in the alga. It is not clear whether the 9-*cis* isomer is converted to the other stereoisomers during ingestion and absorption, or whether it is directly stored in specific tissues, but not in plasma. In this paper, we investigate changes in stereoisomers of β-carotene in plasma and in blood cells of healthy human subjects after ingesting either naturally-occurring β-carotene derived from *D. bardawil* or a synthesized all-*trans* β-carotene preparation.

*To whom correspondence should be addressed at Department of Pediatrics, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569, Japan.

Abbreviations: HPLC, high-performance liquid chromatography; MN, mononuclear cell; RBC; red blood cell.

MATERIALS AND METHODS

Subjects and β-carotene intake. Thirty male volunteers, aged 20–25 years old, were given either 60 mg of a synthesized all-*trans* β-carotene (kindly provided by Nippon Roche K.K., Tokyo, Japan), 60 mg of a naturally-occurring β-carotene derived from *D. bardawil* (Nikken Sohonsha Corporation, Gifu, Japan), or an identical placebo. The preparations were taken orally every morning immediately after breakfast for 44 wk. None of the subjects took any other vitamin supplements or medications before or during the study. The study was performed in a single blind manner. The study protocol was approved by the ethics committee of the hospital, and informed consent was obtained from the subjects themselves.

Blood collection. Blood was collected with EDTA–Na_2 after an overnight fast. The blood samples were centrifuged at 900 rpm for 10 min at room temperature to obtain platelet-rich plasma in the top layer and white blood cells containing red blood cells (RBC) in the bottom layer. The top layer was centrifuged again at 3000 rpm for 10 min to sediment the platelets, which were suspended in a sterile plastic tube with 10 mL of 0.9% NaCl and washed three times with 10 mL of 0.9% NaCl (2 × 5 min and 1 × 10 min). After the final washing, the platelet pellet was resuspended in 2.5 mL of 0.9% NaCl and sonicated for 1 min at 20 kc with a Handy Sonic (Ultrasonic Disruptor UD-201; Tomy Co. Ltd., Tokyo, Japan). RBC and mononuclear cells (MN) were separated by density-gradient centrifugation using a "Lymphoprep®" (Nycomed Pharma AS, Oslo, Norway). Three milliliters of the RBC–MN suspension were slowly placed on the 1.5 mL layer of the medium, and then centrifuged at 1,200 rpm for 30 min at 10°C. Thus, the MN layer was separated from the RBC layer. The RBC pellet was washed three times with 0.9% NaCl (2 × 5 min and 1 × 10 min), and after the final washing the cells were resuspended in an equivalent volume of 0.9% NaCl. The hematocrit of the RBC suspension was also measured for a final correction. The tubes containing MN were filled with 10 mL of 0.9% NaCl and centrifuged at 10°C for

Copyright © 1995 by AOCS Press

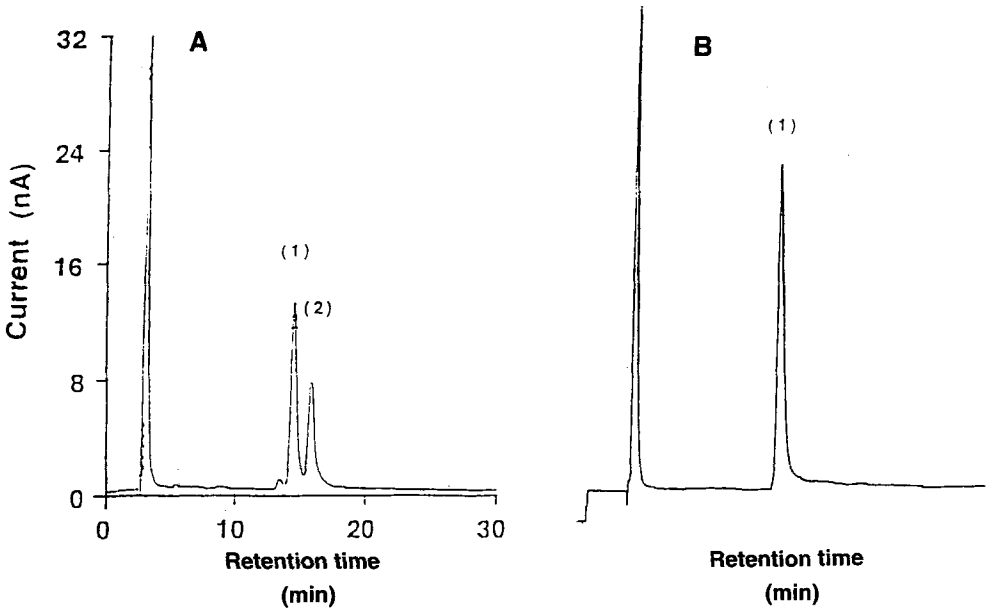


FIG. 1. Chromatograms of extracts from the *Dunaliella* preparation and the synthesized all-*trans* β-carotene. Equal amounts of all-*trans* and 9-*cis* β-carotenes were found in the *Dunaliella bardawil* extract. A, the *D. bardawil* extract; B, the synthesized all-*trans* β-carotene; C, the authentic standards. (1) all-*trans* β-carotene; (2) 9-*cis* β-carotene.

15 min at 1,000 rpm. To remove small amounts of RBC contaminated in the layer, the MN pellet was then washed for 40 s with 2 mL of hypotonic 0.2% NaCl solution, to which 2 mL of 1.6% NaCl was added after 40 s. After refilling the tubes with 10 mL of 0.9% NaCl, centrifugation and decantation at 1,000 rpm for 15 min at 10°C were repeated twice. After the final washing, the pellets were suspended in 2.5 mL of 0.9% NaCl, and the cells were counted with a leukocyte counter (Sysmex Platelet Counter, PL-110; Kobe, Japan). Then, the plasma, platelets, MN, and RBC were assayed for β-carotene in this study.

Analysis of β-carotene and retinol. Determination of β-carotene has been reported previously (9). Briefly, 1 mL of ethanol containing 0.015% butylated hydroxytoluene was added to 0.2 mL of plasma or 0.4 mL of a cell suspension followed by vigorous shaking under nitrogen gas. Five mL of 0.9% NaCl was added to this mixture, which was then centrifuged at 3,000 rpm for 10 min, and 4 mL of the hexane layer was evaporated under a stream of nitrogen. The residue was dissolved in 50 μL of ethanol; a 20 μL aliquot was injected into the high-performance liquid chromatography (HPLC) column for determination of β-carotene, and the 20 μL aliquot from the plasma extract sample was injected into the other HPLC column for retinol determination. The HPLC equipment for β-carotene was an IRICA Σ 871 (IRICA Co. Ltd., Kyoto, Japan) with a Vydac (Hesperia, CA) reverse-phase C_{18} column (Cat. No. 201TP54; 250 × 4.6 mm), and the detector was an IRICA Σ 875 amperometric detector. The standard eluent was methanol/acetonitrile (95:5, vol/vol), including 50 mM $NaClO_4$, and the flow rate was 1 mL/min. Authentic all-*trans* and 9-*cis* β-carotenes were provided by Nippon Roche K.K., and were dissolved in methanol to produce the standard. The concentration of the authentic β-carotene standard was spectrophotometrically determined using an absorption coefficient $E^{1\%}_{1\,cm} = 2620$ at 450 nm for all-*trans* form and $E^{1\%}_{1\,cm} = 2290$ at 446 nm for 9-*cis* form by a Hitachi U-2000 spectrophotometer (10) (Hitachi Co. Ltd., Tokyo, Japan). The HPLC for retinol measurement was a Shimadzu LC-5A (Shimadzu Co. Ltd., Kyoto, Japan) with an IRICA

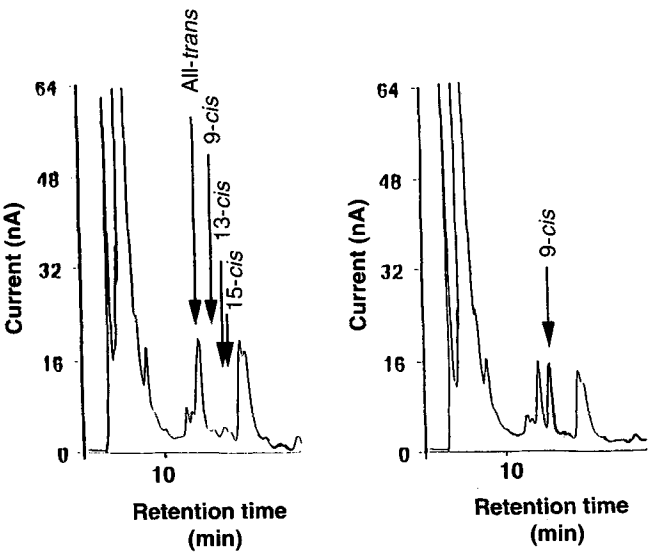


FIG. 2. Chromatograms of extracts from human plasma. Left panel shows clear separation of all-*trans*, 9-*cis*, 13-*cis*, and 15-*cis* β-carotene. Right panel shows the chromatogram added by the authentic 9-*cis* β-carotene. Pure 9-*cis* β-carotene coincided with the presumed 9-*cis* β-carotene. All-*trans*, all-*trans* β-carotene; 9-*cis*, 9-*cis* β-carotene; 13-*cis*, 13-*cis* β-carotene; 15-*cis*, 15-*cis* β-carotene.

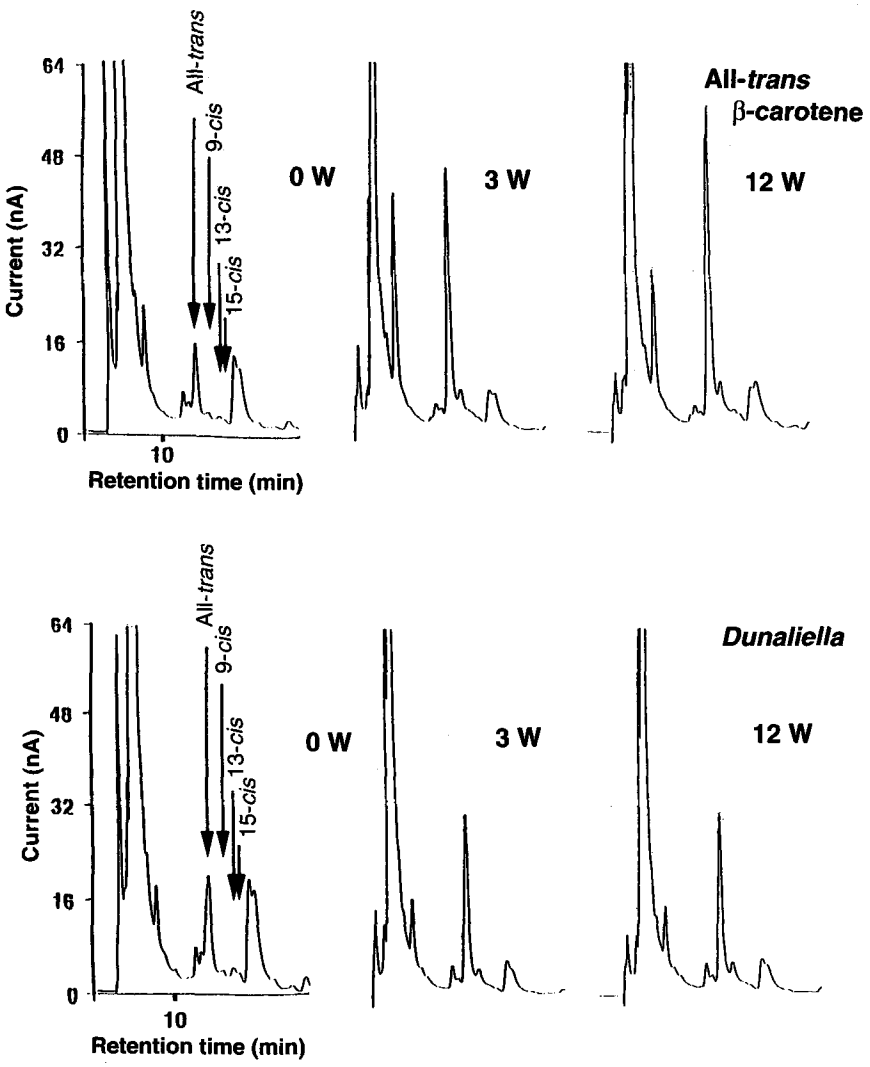


FIG. 3. Typical high-performance liquid chromatography tracings of plasma extracts before and after ingestion of the synthesized β-carotene preparation and the *Dunaliella* preparation. All-*trans*, all-*trans* β-carotene; 9-*cis*, 9-*cis* β-carotene; 13-*cis*, 13-*cis* β-carotene; 15-*cis*, 15-*cis* β-carotene.

RP-18 (4 × 250 mm) column. The detector was a Shimadzu RF-550 fluorescencephotometer (ex: 340 nm, em: 460 nm). The eluent was ethanol/water (95:5, vol/vol), and the flow rate was 0.7 mL/min.

Statistical analysis. All results are expressed as mean ± SE. Statistical significance was assessed at $P < 0.05$ by a repeated measures analysis of variance.

RESULTS

Analysis of administered preparations. Figure 1 shows chromatograms of extracts from two preparations of β-carotene mixture, the naturally-occurring and the synthesized forms, as described in the Materials and Methods section. The naturally-occurring β-carotene derived from *D. bardawil* is approximately 50% all-*trans* β-carotene and 50% 9-*cis* β-carotene, with a very small amount of α-carotene. The synthesized preparation contains only all-*trans* β-carotene, and there were no detectable α-carotene and *cis*-forms. This indicated that no isomerization occurred during sample processing. Figure 2 shows the chromatograms of the plasma extract. The pure additional 9-*cis* β-carotene coeluted with the presumed 9-*cis* β-carotene.

Changes in plasma β-carotene levels after administration of the preparations. Typical chromatograms of plasma extracts before and after carotene administration are shown in Figure 3. The peaks of 13- and 15-*cis* β-carotene were detected after the 9-*cis* form peaked. The basal plasma level of each *cis*-form was found to be only about 10% of the all-*trans* form. In this study, only the all-*trans* and 9-*cis* forms were examined, because administered β-carotenes included predominantly the all-*trans* form and the 9-*cis* form, with much less 13- or 15-*cis* form. The plasma levels reached a maximum after two weeks of administration and plateaued there-

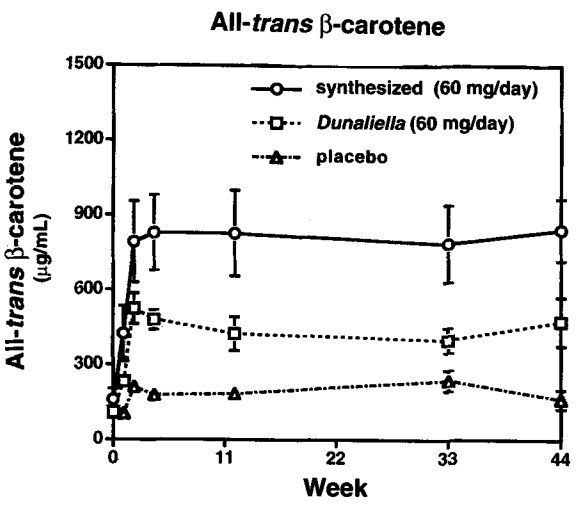


FIG. 4. Changes in plasma levels of all-*trans* and 9-*cis* β-carotenes by administration of the *Dunaliella* preparation, the synthesized β-carotene preparation, or a placebo.

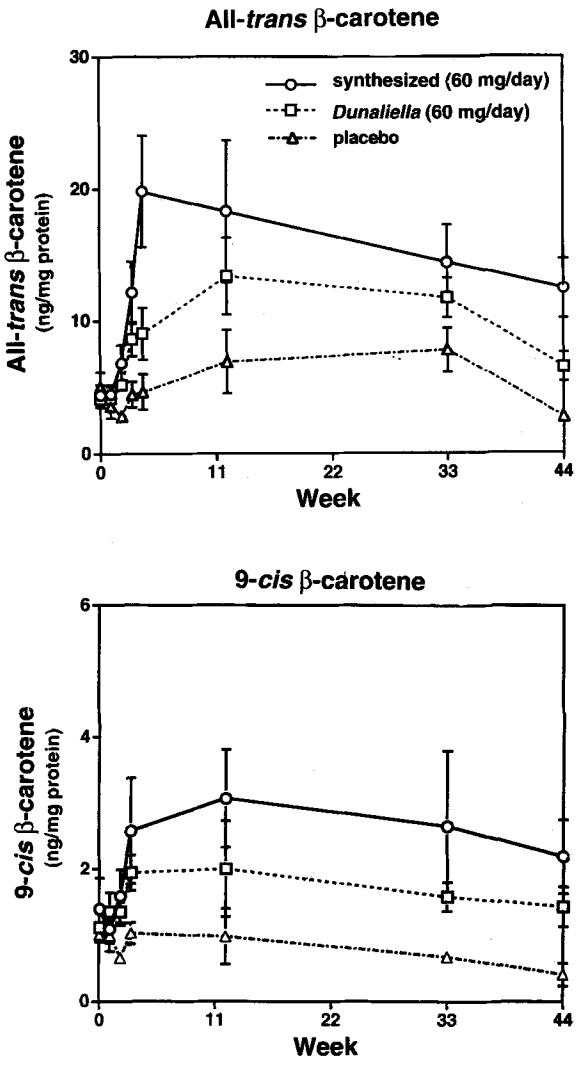


FIG. 5. Changes in platelet content of all-*trans* and 9-*cis* β-carotenes by administration of the *Dunaliella* preparation, the synthesized β-carotene preparation, or a placebo.

after in the subjects who took the β-carotene preparations, and the plasma level in the placebo group showed only seasonal variation (Fig. 4). The all-*trans* β-carotene level in the subjects given the synthesized all-*trans* β-carotene was almost twice that for the *Dunaliella* preparation, indicating that the elevation of plasma level corresponded to the content of the all-*trans* form of both preparations. The plasma level of the 9-*cis* form did not show the same pattern as for the all-*trans* form, because a higher plasma 9-*cis* level was found in the all-*trans* β-carotene group than in the *Dunaliella* group, despite no intake of the *cis*-form in the all-*trans* group and a higher intake of the 9-*cis* form in the *Dunaliella* group.

Changes in β-carotene isomer level in platelets and MN. The ratio of 9-*cis* to all-*trans* in platelets (20%) and MN (25%) was higher than that in the plasma at basal levels before administration. As shown in Figures 5 and 6, all-*trans* β-carotene was also dose-dependently elevated in platelets and MN of the two β-carotene groups. With respect to RBC β-carotenes, the basal level was low and no remarkable changes were observed during and after administration (data not shown). With respect to 9-*cis* β-carotene levels in platelets and in MN, the elevation after administration was marked both for the all-*trans* and *Dunaliella* groups after administration, as compared with the placebo group. Changes in the ratio of 9-*cis* to all-*trans* in platelets and MN in the all-*trans* group were similar to those in the *Dunaliella* group after administration, indicating that increased 9-*cis* levels occurred even in the cells of the all-*trans* group, despite the fact that no *cis*-form existed in the all-*trans* preparation.

Changes in plasma retinol levels after administration with β-carotene preparations. Throughout the experimental periods (Fig. 7), the plasma retinol level did not change in the groups with β-carotenes as compared with the placebo group.

DISCUSSION

Recently, Stahl *et al.* (4) investigated the β-carotene isomers in human serum and tissues and reported that 9-*cis* β-carotene

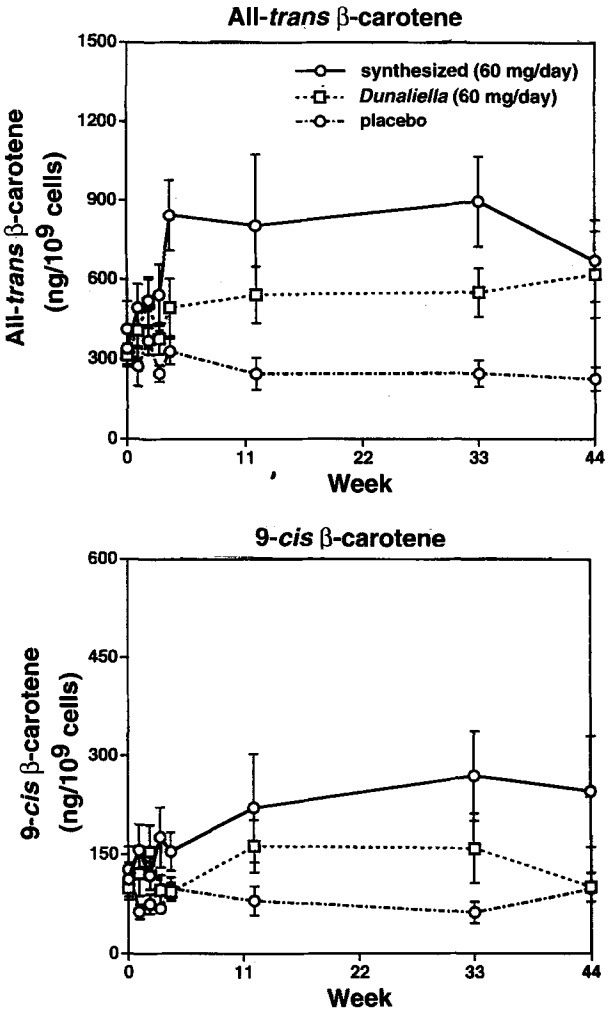


FIG. 6. Changes in mononuclear cell content of all-*trans* and 9-*cis* β-carotenes by administration of the *Dunaliella* preparation, the synthesized β-carotene preparation, or a placebo.

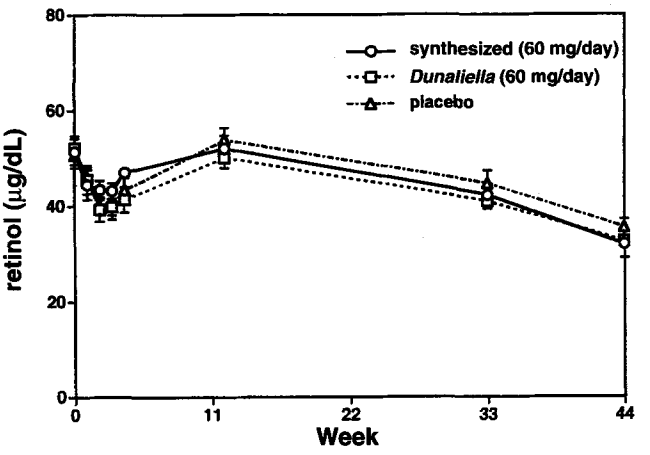


FIG 7. Changes in plasma retinol by administration of the *Dunaliella* preparation, the synthesized β-carotene preparation, or a placebo.

is not detectable in serum although it is present in tissues, especially in liver. They have proposed that the 13-*cis* form, rather than the 9-*cis* form, is the dominant *cis*-isomer in humans. Similar findings were observed in other studies (11,12). Under our HPLC conditions, the 9-*cis* form is detected in plasma, and shows a similar peak to the 13- and 15-*cis* forms. In this study, only changes in the level of the all-*trans* form and 9-*cis* form were detected, because the administered preparation from *D. bardawil* included all-*trans* and 9-*cis*, but much less 13- and 15-*cis* β-carotene and α-carotene. Stahl *et al.* (7) found that the all-*trans* form predominates serum and tissues, and presumed that this is either due to preferential absorption of the all-*trans* form, rapid distribution of the 9-*cis* form into the tissues, or isomerase activity converting the 9-*cis* to the all-*trans* form. With respect to the basal level, a relatively higher ratio of 9-*cis* form to all-*trans* form in the platelets and MN was observed, as compared to that in plasma. This may indicate the accumulation of 9-*cis* in the cells. In addition, after administration with both β-carotene preparations, the elevated plasma and cellular all-*trans* levels depended on the administered dose of the all-*trans* form, while the levels in the 9-*cis* form did not. Different biokinetics may be operative in the body for these isomers. If the cellular level of β-carotene isomers reflects the tissue content, it may be that isomerization of the all-*trans* form to the 9-*cis* form occurs *in vivo*, because a higher level of the 9-*cis* form was observed in MN and platelets after administration with only the synthesized all-*trans* β-carotene, and the level was comparable to that of the *Dunaliella* preparation, which contained a relatively large amount of 9-*cis* isomer.

These findings suggest that 9-*cis* isomer may not be absorbed through the intestine, probably because of specific discrimination mechanism for geometrical isomers. However, it is unlikely that 9-*cis* isomer is absorbed, but very rapidly distributed, into tissues or that isomerase produces all-*trans* β-carotene from the 9-*cis* form. The likely explanation is that isomerization of all-*trans* to 9-*cis* occurs in the body either during or after absorption. The latter hypothesis comes from our finding that, in plasma and blood cells, the 9-*cis* level rose even after the administration of the synthesized all-*trans* form, while changes in the all-*trans* level after administration were quite dose-dependent. To confirm this hypothesis, administration of the purified 9-*cis* isomer needs to be investigated.

ACKNOWLEDGMENTS

We gratefully thank Nikken Sohonsha Corporation (Gifu, Japan) and Nippon Roche K.K. (Tokyo, Japan) for their financial support. We also thank Dr. H. Takenaka (MicroAlgae Corporation, Gifu, Japan) for his useful comments.

REFERENCES

1. Peto, R., Doll, R., Buckley, J.D., and Sporn, M.B. (1981) Can Dietary Beta-Carotene Materially Reduce Human Cancer Rates? *Narute 290*, 201–208.

2. Ziegler, R.G. (1989) A Review of Epidemiologic Evidence That Carotenoids Reduce the Risk of Cancer, *J. Nutr. 119*, 116–122.
3. Krinsky, N.I., Russett, M.D., Handelman, G.J., and Snodderly, D.M. (1990) Structural and Geometrical Isomers of Carotenoids in Human Plasma, *J. Nutr. 120*, 1654–1662.
4. Stahl, W., Schwarz, W., Sundquist. A.R., and Sies, H. (1992) *Cis-trans* Isomer of Lycopene and β-Carotene in Human Serum and Tissues, *Arch. Biochem. Biophys. 294*, 173–177.
5. Stahl, W., Sundquist, A.R., Hanusch, M., Schwarz, W., and Sies, H. (1993) Separation of β-Carotene and Lycopene Geometrical Isomers in Biological Samples, *Clin. Chem. 39*, 810–814.
6. Jensen, C.D., Howes, T.W., Spiller, G.A., Pattison, T.S., Whittam, J.J., and Scala, J. (1987) Observations on the Effects of Ingesting *cis*- and *trans*-beta-Carotene Isomers on Human Serum Concentrations, *Nutr. Rep. Int. 35*, 413–422.
7. Stahl, W., Schwarz, W., and Sies, H. (1993) Human Serum Concentrations of all-*trans* β-Carotene and α-Carotene But Not 9-*cis* β-Carotene Increase upon Ingestion of a Natural Isomer Mixture Obtained from *Dunaliella salina* (Betatene), *J. Nutr. 123*, 847–851.
8. Tamai, H., Murata, T., Morinobu, T., Manago, M., Takenaka, H., Hayashi, K., and Mino, M. (1993) Bioavailability of β-Carotene in a Carotenoid Preparation Derived from *Dunaliella bardawil* in Human Male Adults, *Annals N.Y. Acad. Sci. 691*, 238–240.
9. Murata, T., Tamai, H., Morinobu, T., Manago, M., Takenaka, A., Takenaka, H., and Mino, M. (1992) Determination of β-Carotene in Plasma, Blood Cells and Buccal Mucosa by Electrochemical Detection, *Lipids 27*, 840–843.
10. Ben-Amotz, A., Mokady, S., Edelsten, S., and Avron, M. (1989) Bioavailability of a Natural Isomer Mixture as Compared with Synthetic all-*trans*-Carotene in Rats and Chicks, *J. Nutr. 119*, 1013–1019.
11. Rushin, W.G., Catignani, G.L., and Schwartz, S.J. (1990) Determination of β-Carotene and Its *cis* Isomer in Serum, *Clin. Chem. 36*, 1986–1989.
12. Granado, F., Olmedilla, B., Blanco, I., and Rojas-Hidalgo, E. (1991) An Improved HPLC Method for the Separation of Fourteen Carotenoids, including 15-/13- and 9-*cis*-Carotene Isomers, Phytoene and Phytofluene, *J. Liqu. Chromatogr. 14*, 2457–2475.

[Received September 12, 1994, and in revised form March 4, 1995; Revision accepted March 21, 1995]

Sesamin (a compound from sesame oil) Increases Tocopherol Levels in Rats Fed *ad libitum*

Afaf Kamal-Eldin*, Dan Pettersson and Lars-Åke Appelqvist

Department of Food Science, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden

ABSTRACT: Six groups of rats were fed diets low, but adequate, in α-tocopherol but high in γ-tocopherol. The six diets differed only in their contents (0, 0.25, 0.5, 1.0, 2.0, and 4.0 g/kg, respectively) of sesamin, a lignan from sesame oil. After four weeks of *ad libitum* feeding, the rats were sacrificed and the concentrations of α- and γ-tocopherols were measured in the plasma, livers, and lungs. Sesamin-feeding increased γ-tocopherol and γ-/α-tocopherol ratios in the plasma ($P < 0.05$), liver ($P < 0.001$), and lungs ($P < 0.001$). The increase was non-significant for α-tocopherol. Thus, sesamin appears to spare γ-tocopherol in rat plasma and tissues, and this effect persists in the presence of α-tocopherol, a known competitor to γ-tocopherol. This suggests that the bioavailability of γ-tocopherol is enhanced in phenol-containing diets as compared with purified diets.

Lipids 30, 499–505 (1995).

Sesame seed (*Sesamum indicum*, Linn., Pedaliaceae) is known for its high nutritional value, and for having high oil (*ca.* 50%) and protein (20–25%) content (1). Sesame oil is characterized by a very high oxidative stability compared with other vegetable oils (2,3). The oil is composed of *ca.* 98% glycerides and 1.5–2% unsaponifiables (4,5). The fatty acid composition of sesame oil is palmitic (7–12%), stearic (3.5–6%), oleic (35–50%), and linoleic acid (35–50%) (4,6,7). Two lignan-type compounds, sesamin and sesamolin, are the major constituents of sesame oil unsaponifiables (5,8). Sesamol (a sesamolin derivative) can be present in sesame seeds and/or oils in very small amounts. Other lignans and sesamol are also present in sesame seeds and/or oils in very small amounts as aglycones, but are present in considerable amounts in the seeds as glucosides (9–14). The structures of all sesame seed lignans are shown in Figure 1, and their systematic names and levels in sesame seeds are presented in Table 1. Crude sesame oil also contains *ca.* 500–700 mg tocopherols per kg, which are about 97% γ-tocopherol (γ-T) (5).

Sesame seed lignans were reported to be responsible for many unique chemical and physiological properties of sesame oil (2,9,15–21). It is of special interest to note that they have antioxidant and antimutagenic properties (9,22). Sesamin has no antioxidant activity in itself, but it is possible that its metabolites can act as antioxidants *in vivo*. Recently, Yamashita *et al.* (23) suggested that certain antioxidants in sesame, other than tocopherols, suppressed senescence in mice. Sesamin also caused a significant increase in γ-T levels in the plasma and liver of rats (24). In their experiment, Yamashita *et al.* (24) studied the effect of addition of sesame seed, sesamin, and sesaminol (a transformation compound from sesamolin in refined sesame oil) to rat diets containing

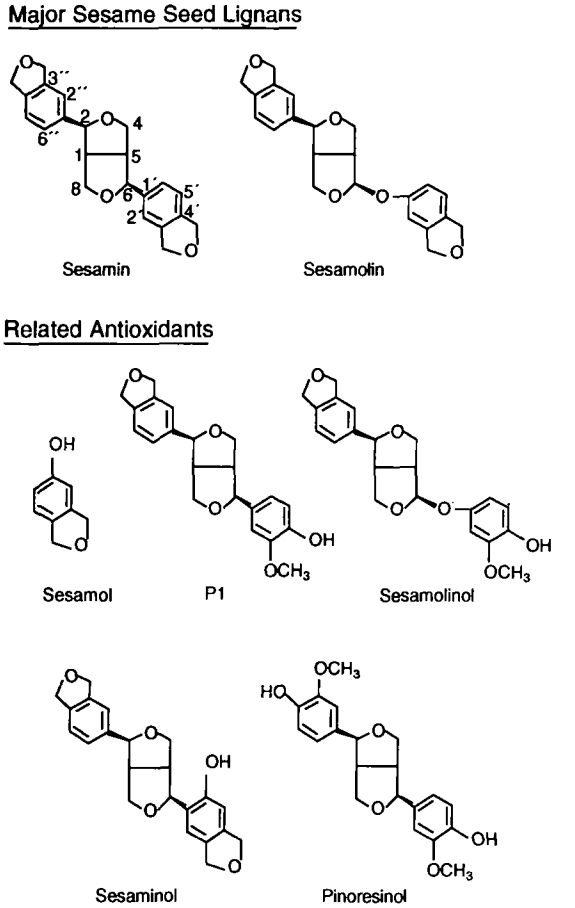


FIG. 1. The chemical structures of sesamin and related compounds in sesame seeds.

*To whom correspondence should be addressed at Dept. of Food Science, Swedish University of Agricultural Sciences, Box 7051, 750 07 Uppsala, Sweden.

Abbreviations: α-T, α-tocopherol; γ-T, γ-tocopherol; GTL, γ-tocopherol levels; HPLC, high-performance liquid chromatography; SL, sesamin levels.

Copyright © 1995 by AOCS Press

TABLE 1
Trivial and Systematic Names and Levels[a] of Lignans and Lignan Glucosides in Sesame Seeds (*Sesamum indicum*, Linn.)

Trivial names	Systematic names	Weight % in seed	Reference number
Lignans			
Sesamin	2,6-*bis*-(3,4-methylenedioxy phenyl)-*cis*-3,7-dioxabicyclo-[3.3.0]-octane	0.2–0.5	9
Sesamolin	2-(3,4-methylenedioxy phenoxy)-6-(3,4-methylenedioxy phenyl)-*cis*-3,7-dioxabicyclo-[3.3.0]-octane	0.2–0.3	9
Sesamol	3,4-methylenedioxy phenol	0.0004	10
P1	2-(3-methoxy-4-hydroxy phenyl)-6-(3,4-methylenedioxy phenyl)-*cis*-3,7-dioxabicyclo-[3.3.0]-octane	0.002	11
Sesamolinol (P2)	2-(3-methoxy-4-hydroxy phenoxy)-6-(3,4-methylenedioxy phenyl)-*cis*-3,7-dioxabicyclo[3.3.0]-octane	0.006	11
Sesaminol (P3)	2-(3,4-methylenedioxy-6-hydroxy phenyl)-6-(3,4-methylenedioxy phenyl)-*cis*-3,7-dioxabicyclo [3.3.0]-octane	0.002	11
Pinoresinol	2,6-*bis*-(3-methoxy-4-hydroxy phenyl)-*cis*-3,7-dioxabicyclo[3.3.0]-octane	—	12
Lignan glucosides		total *ca.* 1%	12
—	Sesaminol 2′-O-ß-D-glucopyranoside	—	13
—	Sesaminol 2′-O-ß-D-glucopyranosyl(1→2)-ß-D-glucopyranoside	—	13
—	Sesaminol 2′-O-ß-D-glucopyranosyl(1→2)-O-[β-D-glucopyranosyl(1→6)]-ß-D-glucopyranoside	—	13
KP1	Pinoresinol 4′-O-ß-D-glucopyranosyl(1→6)-ß-D-glucopyranoside	—	12
KP2	Pinoresinol 4′-O-ß-D-glucopyranosyl(1→2)-ß-D-glucopyranoside	—	12
KP3	Pinoresinol 4′-O-ß-D-glucopyranosyl(1→2)-O-[ß-D-glucopyranosyl(1→6)]-ß-D-glucopyranoside	—	14

[a]The levels of total lignans and lignan glucosides in sesame seeds can range from 1–1.5%.

practically no α-tocopherol (α-T) but being rich in γ-T. It has been reported that γ-T can be present in plasma at fairly high concentrations in cases of α-T deficiency (25,26). On the other hand, with increased intake, α-T displaces γ-T in serum (27,28) as well as in red blood cells, platelets, and lymphocytes (29).

As mixed diets for humans and animals generally contain both α-T and γ-T as major dietary tocopherols, we considered it relevant to study the effects of dietary sesamin on the retention of γ-T in the rat in the presence of low, but adequate, levels of α-T (30).

MATERIALS AND METHODS

Chemicals and reagents. Sesamin, DL–γ-T and groundnut oil were gifts from Takemoto Oil & Fat Co., Ltd. (Gamagori Aichi, Japan), F. Hoffmann-La Roche (Basel, Switzerland), and from Aarhus Olie (Aarhus, Denmark), respectively. The authentic α- and γ-T used as external standards in high-performance liquid chromatography (HPLC) analyses were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade and were used without further purification.

Animals, diets, and study design. Thirty-six male, 25-day-old Sprague-Dawley rats (B & K Universal, Sollentuna, Sweden) weighing on average 70 g, were used. The rats were housed individually in wire-bottom cages in a room kept at 25°C and 50% relative humidity. A 12-h light (07:00–19:00)/dark (19:00–07:00) cycle was used. Six groups, of six rats each, were fed the various experimental diets (Table 2). Groundnut oil (containing 13 mg of endogenous α-T and 10 mg of endogenous γ-T per 100 g and fortified with additional γ-T, 40 mg/100 g) was added, at a level of 100 g/kg diet, as a source of energy, essential fatty acids, and vitamin E. Thus, the vitamin E content of the six diets was 13 mg/kg α-T and 50 mg/kg γ-T. Sesamin was the only variable in the diets and was present in diets 1–6 at levels of 0.0, 0.25, 0.50, 1.0, 2.0, and 4.0 g/ kg, respectively. The rats were allowed free access to tap water and were fed experimental diets *ad libitum* for four weeks. Feed was given daily (13:00), and feed wastage was determined at the same time. The rats were weighed every week and the tissues were weighed at sacrifice. This

TABLE 2
Composition of the Basal Experimental Diet[a]

Ingredient	g/kg
Maize starch	570
Casein, vitamin free	200
Groundnut oil[b]	100
Cellulose powder	40
Mineral and trace element premix[c]	40
Sucrose	40
Vitamin premix[d]	10

[a]On dry weight basis, seamin was added to the six diets at levels of 0, 0.25, 0.50, 1.0, 2.0, and 4.0 g/kg diet, respectively.
[b]The groundnut oil had the following fatty acid composition, based on gas chromatographic analysis of the fatty acid methyl esters: 16:0 (12.7%), 18:0 (2.4%), 18:1 (51.3%), 18:2 (31.4%), 20:0 (1.0%), and 20:1 (1.2%). The oil contained (mg/100 g): α-tocopherol (α-T; 13), α-tocotrienol (0.1), ß-tocopherol (0.4), γ-tocopherol (γ-T; 10) and δ-tocopherol (δ-T; 0.6). The oil was supplemented with additional γ-T (40 mg/100 g). Thus, each diet contained 13 and 50 mg/kg of α-T and γ-T, respectively.
[c]The composition of mineral and trace element premix (g/kg) was: Ca, 220; P, 58; Mg, 5; Na, 79; I, 0.065; Se, 0.010; Fe, 2; Co, 0.020; Cu, 0.600; Mn, 2.50; Zn, 3.00.
[d]The composition of the vitamin premix (g/kg) was: vitamin A (retinol), 0.5835; vitamin B_1, 2.57; vitamin B_2, 1.09; vitamin B_6, 1.93; vitamin B_{12}, 0.0028; vitamin C, 0.800; vitamin D_3 (cholecalciferol), 0.0106; pantothenic acid, 3.90; vitamin K_3, 0.430; choline chloride, 188.2; biotin, 0.030; ß-carotene, 0.02; folic acid, 0.27; myo-inositol, 206.4; and nicotinic acid, 8.15.

study was approved by the Ethical Committee for animal experiments of the Uppsala region.

Blood and tissue collections. Rats were deprived of feed for 24 h before sacrifice. Rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital, blood was withdrawn from *vena cava,* and rats were killed by exsanguination. Blood was collected in tubes containing EDTA as anticoagulant, and plasma was isolated following centrifugation (3000 rpm, 4°C, 10 min). The plasma was stored at −20°C until analyzed for tocopherol concentration. The livers and lungs were quickly removed, weighed, immersed in isopropanol (10 mL), and stored at −20°C until analyzed.

Chemical analysis. The tocopherols were extracted from 500 μL portions of the plasma in glass tubes. Ethanol (500 μL) was added to each tube, and the lipids were extracted twice with 2 mL of *n*-hexane after shaking and centrifugation. The lipid extracts were dried over anhydrous sodium sulfate, centrifuged (4000 rpm, 5 min), quantitatively transferred to other tubes, flushed with nitrogen, and sealed and stored at −20°C until analyzed by HPLC within the same or next day. Before HPLC analyses, the hexane extracts were dried under nitrogen and redissolved in 200 μL of hexane.

The livers and lungs were extracted in hexane/isopropanol (3:2, vol/vol) according to Hara and Radin (31) using an Ultra-Turrax homogenizer (Janke and Kunkel GmbH & Co. KG, Breisgau, Germany). Briefly, the livers were homogenized in 100 mL hexane/isopropanol (3:2, vol/vol) and centrifuged at 3000 rpm for 10 min. The lipid extracts were decanted, the tissue was rehomogenized in 50 mL hexane/isopropanol (3:2, vol/vol), and extracts pooled. The extracts were washed twice with aqueous sodium sulfate, and the salt solution was then washed twice with hexane/isopropanol (7:2, vol/vol) (31). The lipid extracts were concentrated under vacuum and transferred to glass tubes containing anhydrous sodium sulfate. The tubes were centrifuged (4000 rpm, 5 min), and the lipid extracts were quantitatively transferred to other tubes. The hexane/isopropanol was evaporated to dryness under nitrogen because α-T has a higher oxidation rate in protic polar solvents. The extracts were then dissolved in hexane, flushed with nitrogen, and sealed and stored at −20°C until analyzed by HPLC within 1–2 d. The solvent was completely evaporated under nitrogen, and the residue was reconstituted in *n*-hexane directly before HPLC analyses.

The tocopherol levels in the plasma and tissues were quantified by HPLC analysis on a Hibar pre-packed LiChrosorb NH_2 column (25 cm × 4 mm i.d., particle size 5 μm; E. Merck). The mobile phase was *n*-heptane/methyl-tertiary butyl ether/tetrahydrofuran/methanol (79:20:1:0.1, by vol) at a flow rate of 1.0 mL/min. The HPLC system consisted of a Merck-Hitachi HPLC L-6200A Intelligent pump, a 20 μL injection loop, and a Merck F-1050 Fluorescence spectrophotometer. The peaks were detected at an excitation wavelength of 295 nm and an emission wavelength of 320 nm, and were recorded using an HP 3396A integrator (Hewlett Packard, Avondale, PA). The tocopherol peaks were identified and quantified against authentic tocopherols used as external standards.

Statistical analysis. Statistical analyses were performed by using the Statistical Analysis System (32). Linear regression analyses of tocopherol concentrations (Y) against sesamin level (X) were done by using the regression procedure (PROC REG) and analysis of variance by using the general linear model with level of sesamin as the only main effect.

RESULTS

The relationships between the sesamin levels in the diets and the α- and γ-T concentrations in the plasma, livers, and lungs of the experimental rats are shown in Figure 2. Positive regression slopes were obtained for both α- and γ-T, showing an increasing effect with increasing sesamin levels in the diets.

For γ-T, the following regression equation was obtained for the plasma: GTL (γ-T level) = 0.270 + 0.214 SL (sesamin level), with a correlation coefficient (r) of 0.60. The intercept (0.270) shows γ-T level (μmole/L plasma) in the control group, and the slope (0.214) shows the rate of increase in γ-T level with increasing sesamin level (SL, g/kg diet) in the experimental groups. The regression equations for γ-T in the liver and lungs were: GTL = 2.913 + 2.072 SL (r = 0.839) and GTL = 1.898 + 2.663 SL (r = 0.86), respectively. The regression analysis procedure showed significant correlations between SL and GTL in plasma ($P < 0.005$), liver ($P < 0.001$), and lungs ($P < 0.001$). On the other hand, the relationships between the sesamin level in the diet (SL) and α-T levels in the plasma, liver, and lungs were not statistically significant (Fig. 2).

The effects of increasing sesamin concentration on the means of α-T, γ-T, and α-T + γ-T levels and on the ratios of γ-T/α-T in the plasma and tissues of experimental rats were assessed by analysis of variance (Table 3). Plasma γ-T concentrations generally increased from 0.06 to 1.07 μmol/L, whereas α-T concentrations varied between 3.06 and 4.79 mmol/L. The concentration of γ-T in the plasma was significantly increased ($P < 0.05$) by sesamin level, whereas the increase in α-T concentration was not statistically significant. The sesamin feeding did not change total body weights nor weights of livers and lungs of experimental rats. In the livers, γ-T increased from 2.12 to 11.03 nmol/g fresh weight ($P < 0.001$) as a result of increasing dietary sesamin. Although α-T concentrations were not significantly changed, they varied between 16.96 and 27.03 nmol/g fresh weight. Similar results were obtained in the lungs, where γ-T concentrations increased from 1.55 to 13.07 nmol/g fresh weight ($P < 0.001$) and α-T concentrations did not change significantly.

DISCUSSION

In this study, the rat diets were rich in γ-T and contained an adequate level of α-T, as judged from the results of Hakkarinen *et al.* (30). The minimum vitamin E requirement for rat survival was reported as 7 mg DL-α-tocopherylacetate per kg

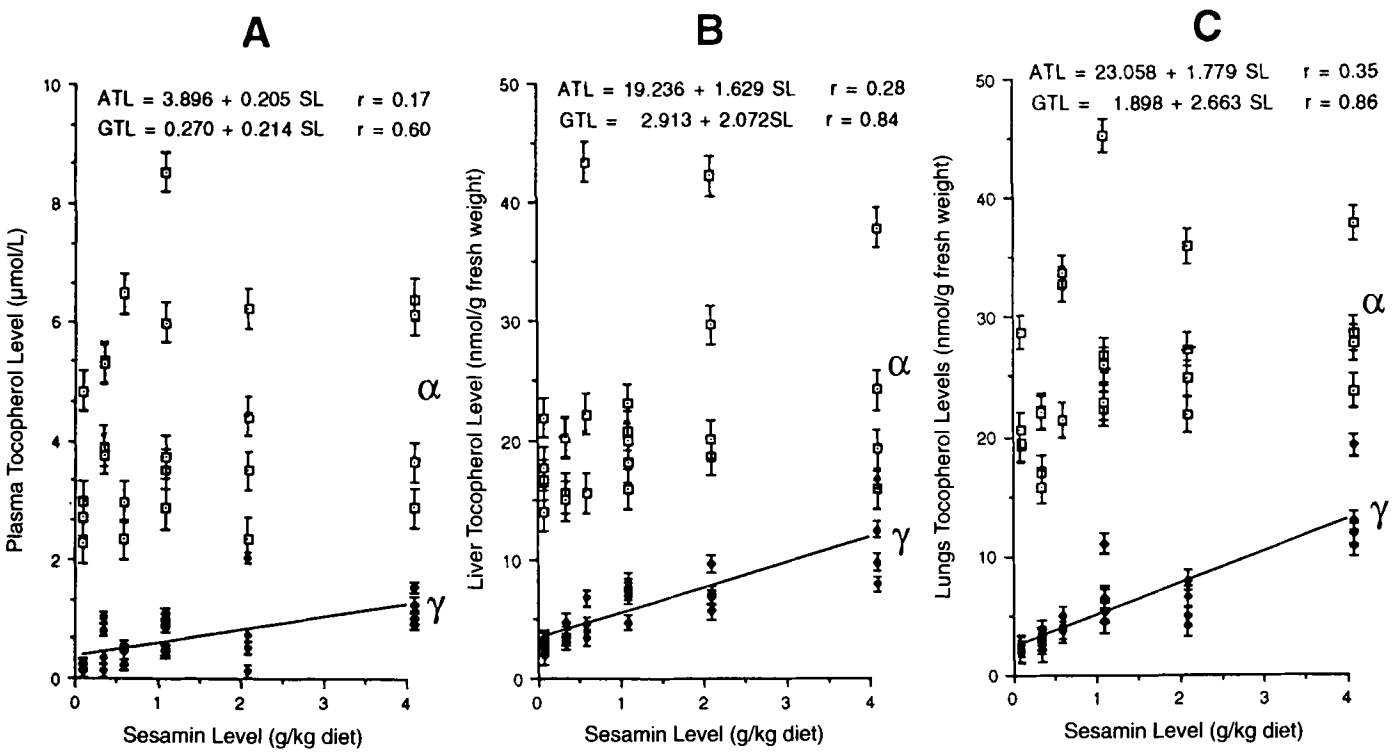


FIG. 2. Linear relationships between α- and γ-tocopherol levels (ATL and GTL, respectively) in rat plasma (A), liver (B), and lungs (C), and sesamin level (SL) in the diet (g/kg). The points show the tocopherol levels for 3–5 rats per sesamin level (group); r denotes the correlation coefficients: the correlations were nonsignificant in the case of α-Tocopherol (α), but significant in the case of γ-tocopherol (γ) (plasma, $P < 0.005$; liver and lungs, $P < 0.001$).

feed when the selenium content of the diet is 6.6 μg/kg and the vitamin E/polyunsaturated fatty acid ratio is 0.45 (30). This experimental approach was introduced to explore if the presence of the adequate α-T levels will mask the reported effect of sesamin on γ-T retention (24). The experiment was designed so that sesamin content was the only differing variable in the experimental diets. Groundnut oil was chosen because of its resemblance to sesame oil in fatty acid composition and for the fact that it contains only α-T and γ-T as major tocopherols (Refs. 4, 6, Table 2).

TABLE 3
Concentrations of α- , γ-, and α + γ-Tocopherols and γ-/α-Tocopherol Ratios in Plasma (μmol/L) and Liver and Lungs (nmol/g wet weight) of Rats Fed a Basal Diet with Increasing Levels of Sesamin[a]

Sesamin level (g/kg diet)	0	0.25	0.50	1.0	2.0	4.0	*P* value for analysis of variance ($P <$)
Number of observations	4	4	3	5	4	4	
Plasma							
α-Tocopherol	3.1 ± 0.56	4.4 ± 0.43	3.8 ± 1.29	4.8 ± 1.04	4.0 ± 0.82	4.6 ± 0.88	N.S.
γ-Tocopherol	0.1 ± 0.03^{b}	0.4 ± 0.21^{b,d}	0.3 ± 0.09^{b,c}	0.6 ± 0.13^{b,d}	0.7 ± 0.41^{c,d}	1.1 ± 0.14^{d}	0.05
α + γ-Tocopherol	3.1 ± 0.54	4.9 ± 0.46	4.1 ± 1.37	5.4 ± 1.11	4.7 ± 1.21	5.7 ± 1.01	N.S.
γ-Tocopherol/α-tocopherol	0.0 ± 0.01^{b}	0.1 ± 0.05^{b,c}	0.1 ± 0.02^{b,c}	0.1 ± 0.03^{c,d}	0.1 ± 0.07^{b,d}	0.2 ± 0.23^{d}	0.05
Liver							
α-Tocopherol	17.0 ± 1.64	17.1 ± 1.45	26.5 ± 8.41	18.9 ± 1.21	27.0 ± 5.45	23.6 ± 4.83	N.S.
γ-Tocopherol	2.1 ± 0.31^{b}	3.0 ± 0.36^{b}	4.2 ± 0.98^{b,c}	6.3 ± 0.60^{c}	6.6 ± 0.85^{c}	11.0 ± 1.90^{d}	0.001
α + γ-Tocopherol	19.1 ± 1.91	20.2 ± 1.42	30.7 ± 9.39	25.2 ± 1.74	33.6 ± 6.26	34.7 ± 6.56	N.S.
γ-Tocopherol/α-tocopherol	0.1 ± 0.01^{b}	0.2 ± 0.03^{b,c}	0.2 ± 0.01^{b,c}	0.3 ± 0.02^{d}	0.3 ± 0.02^{c,d}	0.5 ± 0.05^{e}	0.001
Lungs							
α-Tocopherol	21.3 ± 2.26	18.6 ± 1.67	28.7 ± 3.95	28.1 ± 4.25	26.9 ± 3.03	28.9 ± 2.98	N.S.
γ-Tocopherol	1.5 ± 0.14^{b}	2.3 ± 0.39^{b,c}	3.5 ± 0.40^{b,d}	6.0 ± 1.23^{d}	5.2 ± 0.89^{c,d}	13.1 ± 1.90^{e}	0.001
α + γ-Tocopherol	22.9 ± 2.35^{b}	20.9 ± 1.84^{b}	32.2 ± 4.05^{b,c}	34.1 ± 5.33^{c}	32.1 ± 3.41^{b,c}	42.0 ± 3.70^{c}	0.01
γ-Tocopherol/α-tocopherol	0.1 ± 0.01^{b}	0.1 ± 0.02^{b,c}	0.1 ± 0.02^{b,c}	0.2 ± 0.02^{c}	0.2 ± 0.03^{c}	0.5 ± 0.07^{d}	0.001

[a]Levels of α- and γ-tocopherols measured by HPLC relative to external standards. Values represent means ± SEM (n = 3–5). The composition of the diets is as in Table 2.

^{b,c,d,e}Values within each row not sharing a common superscript letter are statistically different at $P < 0.05$. N.S., not significant.

In the United States, the daily dietary supply of γ-T is approximately twice that of α-T (33,34). Earlier studies showed that the biological activity of γ-T is only about 10–35% of that of α-T (26–28,35,36). Hence, α-T is recognized as the most important natural lipophilic antioxidant in biological systems (36–39). Both isomers (α-T and γ-T) are absorbed to the same extent from the gastrointestinal tract (25,35,40–43), but γ-T is cleared from the tissues at a considerably faster rate than α-T (44,45). Upon feeding equal amounts of the two isomers, both tocopherols were found to increase similarly in the plasma up to 12 h. After 24 h, the level of γ-T decreased drastically, while that of α-T remained almost unchanged (41,43,46). The latter is preferentially bound to the cellular membranes of the liver and to the transporting proteins, while γ-T is excreted through bile without combining with the transporting protein (26,41,43,47). Comparative studies in humans using deuterium-labeled γ-T also showed that there is no discrimination between γ-T and α-T during absorption and secretion in the chylomicrons, but subsequently there is a preferential enrichment of the very low density lipoprotein with α-T (48). All animal studies on relative tocopherol bioavailabilities were conducted using purified diets which contained no antioxidants other than the tocopherols. To date, there is no knowledge about the relative bioavailabilities of the different tocopherols in mixed diets containing other antioxidants, such as plant phenols (49).

Our results are in agreement with previous findings that sesamin-feeding increases γ-T levels in rat plasma and in liver (24). Moreover, we have demonstrated that the effect persists even in the presence of α-T, which is a known competitor to γ-T expression (27–29). In addition, we observed that sesamin induced almost equal increases in the α-T levels in rat plasma, liver, and lungs, although these increases were not statistically significant, perhaps due to the large within-group variation in the levels of this tocol in the plasma and tissues.

The sparing effect of sesamin on vitamin E may explain the observation that some antioxidants, other than tocopherols, in sesame suppressed senescence in mice (23). The resulting increase in total vitamin E bioavailability may also explain some interesting early observations. Tobin (15) reported that daily injection of sesame oil to adrenalectomized female rats increased the number of successful pregnancies, prolonged the survival time of those animals having a successful gestation, and increased their ability to rear young. This effect might have been related to high vitamin E bioavailability as vitamin E is well known to increase fertility in vitamin E-deficient rodents and other animals (50,51). Chou and Marlatt (16) found that sesame oil produced better carotene utilization (a factor of 1.84) than did soybean (1.00) and peanut oils (0.68) when fed to rats. At that time, the effect was surprising as the sesame oil meal contained 11.5 mg tocopherols/kg diet and the soybean oil meal had 27.5 mg /kg. Sesamol was also mentioned to counteract fish oil toxicity to chicks in a similar manner to the antioxidants α-T and x,x′-diphenyl-p-phenyl-enediamine (17).

There is but one report on the absorption of sesamin in rats, where only 0.15% of the fed sesamin was recovered in the lymph during 24 h, suggesting limited absorption (20). Around 15–19% was excreted in the feces in rats fed a purified diet and *ca.* 34% in rats fed a nonpurified diet. The mechanism by which sesamin acts is unknown, but at least two possibilities exist. The mechanism may involve synergistic interaction with γ-T (a regeneration mechanism) in a similar way to other antioxidant synergists, like ascorbic acid. Another possibility is that sesamin may compete with γ-T in its oxidation and/or clearance. Lignans having the methylenedioxyphenyl (1,3-benzodioxole) function are known inhibitors of the mixed function oxidases associated with the endoplasmatic reticulum of the microsomes (52). Many mixed function oxidases are active in the metabolism of lipids, steroids, and in the compounds foreign to the metabolic network (53). Thus, sesamin and related compounds may be regarded as xenobiotic substances by the mixed function oxidases, and their further metabolism may competitively inhibit the activity of these enzymes toward the lipids, sterols, tocopherols, etc.

The sparing of α- and γ-T by sesamin may be of nutritional and physiological significance. A hamburger with a sesame bun, rather common in urban western diets, adds *ca.* 10 mg sesamin to the diet. The metabolism of sesamin in the rat was reported to occur, through the mixed function oxidase enzymes, *via* oxidation of the methylene dioxy function, resulting in a 1,2-diphenol (51), which is expected to have antioxidant properties, at least *in vitro*. Human diets are rich in antioxidant phenols, which may have comparable effects to that of sesamin (49). As sesamin feeding caused a high relative increase in γ-T in rats, we postulate that the bioavailability of γ-T may have been underestimated in the previous studies performed using purified diets (26–28,35,36). The metabolic interaction(s) of γ-T with other food or feed constituents with an antioxidant function needs to be studied further.

ACKNOWLEDGMENTS

We thank Dr. R.V. Cooney (Cancer Research Center, University of Hawaii, Honolulu, HI) for discussions on γ-T and sesame oil during a sabbatical stay (LÅA) at University of Hawaii and for critical reading of the manuscript; Prof. J. Hakkarainen (Department of Clinical Nutrition, this University) for discussions in the preparation for this experiment; Lotta Wall (from this department) for technical assistance; Takemoto Oil & Fat Co., Ltd. (Gamagori Aichi, Japan) and F. Hoffmann-La Roche (Basel, Switzerland) for the sesamin and DL-γ-tocopherol gifts, respectively; and Aarhus Olie (Aarhus, Denmark) for the gift of groundnut oil. The International Science Program in Chemical Sciences (IPICS, Uppsala University) is thanked for extended support to A. Kamal-Eldin.

REFERENCES

1. Weiss, E.A. (1983) *Oilseed Crops*, pp. 292–294, Tropical Agricultural Series, Longman, London.
2. Budowski, P., and Markely, K.S. (1951) The Chemical and Physiological Properties of Sesame Oil, *Chem. Revs. 48*, 125–151.
3. Budowski, P. (1964) Recent Research on Sesamin, Sesamolin and Related Compounds, *J. Am. Oil Chem. Soc. 41*, 280–285.

4. Kamal-Eldin, A., and Appelqvist, L-Å. (1994) Variations in Fatty Acid Composition of the Different Acyl Lipids in Seed Oils from Four *Sesamum* Species, *J. Am. Oil Chem. Soc. 71*, 135–139.
5. Kamal-Eldin, A., and Appelqvist, L-Å. (1994) Variations in the Composition of Sterols, Tocopherols and Lignans in Seed Oils from Four *Sesamum* Species, *J. Am. Oil Chem. Soc. 71*, 149–156.
6. Kamal-Eldin, A., Yousif, G., Iskander, G.M., and Appelqvist, L-Å. (1992) Seed Lipids of *Sesamum Indicum*, L. and Related Wild Species in Sudan. I. Fatty Acids and Triacylglycerols, *Fat Sci. Technol. 94*, 254–259.
7. Spencer, G.F., Herb, S.F., and Gormisky, P.J. (1976) Fatty Acid Composition as a Basis for Identification of Commercial Fats and Oils, *J. Am. Oil Chem. Soc. 53*, 94–96.
8. Kamal-Eldin, A., Appelqvist, L-Å., and Yousif, G. (1994) Lignan Analysis in Seed Oils from Four *Sesamum* Species: Comparison of Different Chromatographic Methods, *J. Am. Oil Chem. Soc. 71*, 141–147.
9. Osawa, T. (1992) Phenolic Antioxidants in Dietary Plants as Antimutagenes, in *Phenolic Compounds in Food and Their Effects on Health. II. Antioxidants and Cancer Prevention* (Hang, M-T., Ho, C-T., and Lee, C.Y., eds.), ACS Symposium Series 507, American Chemical Society, Washington D.C., pp. 135–149.
10. Fukuda, Y., Nagata, Y., Osawa, T., and Namiki, M. (1986) Chemical Aspects of the Antioxidative Activity of Roasted Sesame Seed Oil and the Effects of Using the Oil for Frying, *Agric. Biol. Chem. 50*, 857–862.
11. Fukuda, Y., Osawa, T., Namiki, M., and Ozaki, T. (1985) Studies on Antioxidative Substances in Sesame Seed, *Agric. Biol. Chem. 49*, 301–306.
12. Katsuzaki, H., Kawasumi, M., Kawakishi, S., and Osawa, T. (1992) Structures of Novel Antioxidative Lignan Glucosides Isolated from Sesame Seed, *Biosci. Biotech. Biochem. 56*, 2087–2088.
13. Katsuzaki, H., Kawakishi, S., and Osawa, T. (1994) Sesaminol Glucosides in Sesame Seeds, *Phytochemistry 35*, 773–776.
14. Katsuzaki, H., Kawakishi, S., and Osawa, T. (1993) Structure of Novel Antioxidative Lignan Triglucoside Isolated from Sesame Seed, *Heterocycles 36*, 933–936.
15. Tobin, C.E. (1941) Effects of Adrenolactomy on Pregnancy and Survival of Untreated and Sesame Oil-treated Rats, *Endocrinology 28*, 419–425.
16. Chou, T.C., and Marlatt, A.L. (1953) Factors in the Chinese Diet Affecting Carotene Utilization, *J. Nutr. 51*, 305–315.
17. Ershoff, B.H. (1960) Effects of Diet on Fish Oil Toxicity in the Rat, *J. Nutrition 71*, 45–53.
18. Singhal, P.C., and Joshi, L.D. (1980) Glycemic and Cholesterolemic Role of Ginger and Til, *J. Sci. Res. Plants Med. 4*, 32–34.
19. Sugano, M., Inoue, T., Koba, K., Yoshida, K., Hirose, N., Shinmen, Y., Akimoto, K., and Amachi, T. (1990) Influence of Sesame Lignans on Various Lipid Parameters in Rats, *Agric. Biol. Chem. 54*, 2669–2673.
20. Hirose, N., Inoue, T., Nishihara, K., Sugano, M., Akimoto, K., Shimizu, S., and Yamada, H. (1991) Inhibition of Cholesterol Absorption and Synthesis in Rats by Sesamin, *J. Lipid Res. 32*, 629–638.
21. Shimizu, S., Akimoto, K., Shinmen, Y., Kawashima, H., Sugano, M., and Yamada, H. (1991) Sesamin is a Potent and Specific Inhibitor of Δ^5-Desaturase in Polyunsaturated Fatty Acid Biosynthesis, *Lipids 26*, 512–516.
22. Namiki, M. and Osawa, T. (1986) Antioxidants/Antimutagens in Foods, in *Antiomutagenesis and Anticarcinogenesis Mechanisms* (Shankel, D.M., Hartman, P.E., Kada, T., and Hollaender, A., eds.), Plenum Publishing Corporation, New York, pp. 131–142.
23. Yamashita, K., Kawagoe, Y., Nohara, Y., Namiki, M., Osawa, T., and Kawagishi, S. (1990) Effect of Sesame in the Senescence-Accelerated Mouse, *J. Jpn. Soc. Nutr. Food Sci. 43*, 445–449.
24. Yamashita, K., Nohara, Y., Katayama, K., and Namiki, M. (1992) Sesame Seed Lignans and γ-Tocopherol Act Synergistically to Produce Vitamin E Activity in Rats, *J. Nutr. 122*, 2440–2446.
25. Behrens, W.A., and Madere, R. (1983) Interrelationship and Competition of α- and γ-Tocopherol at the Level of Intestinal Absorption, Plasma Transport and Liver Uptake, *Nutr. Res. 3*, 891–897.
26. Behrens, W.A., and Madere, R. (1987) Mechanism of Absorption, Transport and Tissue Uptake of RRR-α-Tocopherol and d-γ-Tocopherol in the White Rat, *J. Nutr. 117*, 1562–1569.
27. Handelman, G.J., Machlin, L.J., Fitch, K., Weiter, J.J., and Dratz, E.A. (1985) Oral α-Tocopherol Supplements Decrease Plasma γ-Tocopherol Levels in Humans, *J. Nutr. 115*, 807–813.
28. Baker, H., Handelman, G.J., Short, S., Machlin, L.J., Bhagavan, H.N., Dratz, E.A., and Frank, O. (1986) Comparison of Plasma α- and γ-Tocopherol Levels Following Chronic Oral Administration of All-*rac*-α-Tocopheryl Acetate or RRR-α-Tocopheryl Acetate in Normal Adult Male Subjects, *Am. J. Clin. Nutr. 43*, 382–387.
29. Lehmann, J., Rao, D.D., Canary, J.J., and Judd, J.T. (1988) Vitamin E and Relationships Among Tocopherols in Human Plasma, Platelets, Lymphocytes and Red Blood Cells, *Am. J. Clin. Nutr. 47*, 470–474.
30. Hakkarainen, J., Työppönen, J., and Jönsson, L. (1986) Vitamin E Requirement of the Growing Rat During Selenium Deficiency with Special Reference to Selenium Dependent and Selenium Independent Glutathione Peroxidase, *J. Vet. Med. Associ. 33*, 247–258.
31. Hara, A., and Radin, N.S. (1978) Lipid Extraction of Tissues with a Low-Toxicity Solvent, *Anal. Biochem. 90*, 420–426.
32. Statistical Analysis Systems Institute, Inc. (1989) *SAS User's Guide, Version 6, Fourth Edn.*, SAS Institute Inc., Cary, North Carolina.
33. Bieri, J.G., and Evarts, R.P. (1974) Tocopherols and Fatty Acids in American Diets, *J. Am. Diet. Assoc. 62*, 147–151.
34. Lehmann, J., Martin, H.L., Lashley, E.L., Marshall, M.W., and Judd, J.T. (1986) Vitamin E in Foods from High and Low Linoleic Acid, *J. Am. Diet. Assoc. 86*, 1208–1216.
35. Bieri, J.G., and Evarts, R.P. (1974) Vitamin E Activity of γ-Tocopherol in the Rat, Chick and Hamster, *J. Nutr. 104*, 850–857.
36. Dillard, C.J., Gavino, V.C., and Tappel, A.L. (1983) Relative Antioxidant Effectiveness of α-Tocopherol and γ-Tocopherol in Iron-Loaded Rats, *J. Nutr. 113*, 2266–2273.
37. Burton, G.W., Joyce, A., and Ingold, K.U. (1983) Is Vitamin E the Only Lipid-Soluble, Chain-Breaking Antioxidant in Human Blood Plasma and Erythrocyte Membranes? *Arch. Biochem. Biophys. 221*, 281–290.
38. Burton, G.W., and Ingold, K.U. (1986) Vitamin E: Application of the Principles of Physical Organic Chemistry to the Explanation of Its Structure and Function, *Acc. Chem. Res. 19*, 194–201.
39. Diplock, A.T., (1983) The Role of Vitamin E in Biological Membranes, in *Biology of Vitamin E* (Porter, R., and Whelan, J., eds.), pp. 45–55, Pitman, London.
40. Bieri, J.G., and Evarts, R.P. (1974) Gamma-Tocopherol: Metabolism, Biological Activity and Significance in Human Vitamin E Nutrition, *Am. J. Clin. Nutr. 27*, 980–986.
41. Peake, I.R., Windmueller, H.G., and Bieri, J.G. (1972) A Comparison of the Intestinal Absorption, Lymph and Plasma Transport and Tissue Uptake of α- and γ-Tocopherols in the Rat, *Biochem. Biophys. Acta 260*, 679–688.
42. Traber, M.G., Kayden, H.J., Green, J.B., and Green, M.H. (1986) Absorption of Water Miscible Forms of Vitamin E in a

Patient with Cholestasis and in Thoracic Duct-Cannulated Rats, *Am. J. Clin. Nutr. 44*, 914–923.
43. Traber, M.G., and Kayden, H.J. (1989) Preferential Incorporation of α-Tocopherol Versus γ-Tocopherol in Human Lipoproteins, *Am. J. Clin. Nutr. 49*, 517–526.
44. Gloor, U., Wursh, J., Schwieter, U., and Wiss, O. (1966) Resorption, Retention, Verteilung und Stoffwechsel des d,l-α-Tocopherolamines, d,l-N-Methyl-γ-Tocopherolamines und des γ-Tocopherol in Vergleich zum d,l-α-Tocopherol bei der Ratte, *Helv. Chim. Acta 49*, 2303–2312.
45. Peake, I.R., and Bieri, J.G. (1971) Alpha- and Gamma-Tocopherols in the Rat: *in vitro* and *in vivo* Tissue Uptake and Metabolism, *J. Nutr. 101*, 1615–1622.
46. Tran, K., and Chan, A.C. (1992) Comparative Uptake of α- and γ-Tocopherol by Human Endothelial Cells, *Lipids 27*, 38–41.
47. Meydani, M., Cohn, J.S., Macauley, J.B., McNamara, J.R., Blumberg, J.B., and Schaefer, E.J. (1989) Postprandial Changes in the Plasma Concentration of α- and γ-Tocopherols in Human Subjects Fed a Fat-rich Meal Supplemented with Fat-soluble Vitamins, *J. Nutr. 119*, 1252–1258.
48. Traber, M.G., Burton, G.W., Hughes, L., Ingold, K.U., Hidaka, H., Malloy, M., Kane, J., Hyams, J., and Kayden, H.J. (1992) Discrimination Between Forms of Vitamin E by Humans with and Without Genetic Abnormalities of Lipoprotein Metabolism, *J. Lipid Res. 33*, 1171–1182.
49. Shahidi, F., and Wanasundara, J.P.D. (1992) Phenolic Antioxidants, *Crit. Revs. Food Sci. Nutr. 32*, 67–103.
50. Evans, H.M., and Bishop, K.S. (1922) On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction, *Science 56*, 650–651.
51. Steele, C.E., Jeffery, E.H., and Diplock, A.T. (1974) The Effect of Vitamin E and Synthetic Antioxidants in the Growth *in vitro* of Explanted Rat Embryos, *J. Reprod. Fert. 38*, 115–123.
52. Cassida, J.E. (1970) Mixed Function Oxidase Involvement in the Biochemistry of Insecticidal Synergists, *J. Agric. Food Chem. 18*, 753–772.
53. Mason, H.S., North, J.C., and Vanneste, M. (1965) Microsomal Mixed-Function Oxidations: The Metabolism of Xenobiotics, *Fed. Proc. 24*, 1172–1180.

[Received September 6, 1994, and in revised form February 22, 1995; Revision accepted March 21, 1995]

Annexin I in Female Rabbit Reproductive Organs: Varying Levels in Relation to Maturity and Pregnancy

Francis H.C. Tsao[a,*], Xiaoru Chen[a], Xiaoming Chen[a] and Chung-hsin Ts'ao[b]

[a]Department of Pediatrics, Division of Neonatology, University of Wisconsin, Madison, Wisconsin 53792 and [b]Department of Pathology, Northwestern University, Chicago, Illinois 60611

ABSTRACT: The level of annexin I, a 36 kDa calcium-dependent phospholipid-binding protein (36 kDa PLBP) in the reproductive organs of young, mature, and pregnant rabbits was determined immunologically with antibodies raised against purified rabbit lung annexin I. In the cytosolic fractions of the ovary, fallopian tube, uterus, and placenta, annexin I was the only major immunoreactive protein. The reproductive organs appeared to have higher annexin I levels than most nonreproductive organ tissues, except the lung and the spleen which were also rich in annexin I. A small amount of annexin I and a nearly equal amount of its hydrolytic product, a 33 kDa polypeptide, were detected in the amniotic fluid between 21 and 27 days gestation. Structural similarity of annexin I in the reproductive organs and in the lung was suggested by their identical isoelectric point values. Annexin I in the ovary of adult rabbits was 70% higher than that in the respective organ of immature rabbits. The uterus of pregnant rabbits had about 84% higher annexin I contents than that of the nonpregnant rabbits. The placenta had more annexin I per mg cytosolic protein than either the ovary or the uterus during pregnancy. The high concentration of annexin I in the reproductive organs may reflect specific functions of these organs in the reproductive years and during the reproductive cycle.

Lipids 30, 507-511 (1995).

Annexins are a group of calcium-dependent phospholipid-binding proteins (PLBPs) that may play a role in signal transduction, membrane fusion, exocytosis, anti-inflammation, and anti-coagulation (1–6). More than eight annexins have been found in various tissues (7–10). The wide distribution of these proteins suggests that each of them may have a specific role in different tissues and cells. Recently, we observed changes in the levels of annexin I, a 36 kDa calcium-dependent PLBP, during growth of the fetal rabbit lung, corresponding to the development of lung surfactant in the alveolar epithelial type II cells (11). Kaetzel and co-workers (12) demonstrated an increased expression of three other annexins called calcimedins 35α, 35β, and 35γ (corresponding to annexins III, IV, and V, respectively) in the rat ovary and uterus during labor. Myatt and co-workers (13,14) detected annexin I in the human amnion and suggested that this protein might regulate prostaglandin production at childbirth. These observations raised the possibility that annexins may play a role in the development of the reproductive organs and during a reproductive cycle. Aside from possibly being a regulator of prostaglandin production (4,5) that increased greatly in the reproductive organs at birth of a newborn, little is known about annexin I with respect to its role in sexual maturity and pregnancy. As an initial step toward understanding the role of annexin I in reproduction, we determined the levels of annexin I in the reproductive organs of young and mature female rabbits, as well as at different stages of pregnancy, with a polyclonal antibody specific to the rabbit lung annexin I. For comparison, we also used the antibody to determine annexin I in some of the nonreproductive tissues to illustrate the tissue specificity of annexin I expression.

MATERIALS AND METHODS

Animals and preparation of tissue samples. Female New Zealand white rabbits, nonpregnant with different ages or pregnant with known gestational ages, were purchased from Bakkom's Rabbitry (Vroqua, WI). The animals were anesthetized with an intravenous injection of sodium pentobarbital/heparin (20 mg/1000 units per kg body weight). For the pregnant rabbits, tracheotomy was performed, and the lung was ventilated with a respirator. Cesarean section was performed to expose the uterus. Amniotic fluid in each amniotic sac was withdrawn. Fetuses were removed for other studies (11). Following the removal of fetuses, portions of the placenta and uterus were collected from each amniotic sac in a total of 10 g for each tissue. Then, the lung of the doe was lavaged with 20 mL of 0.9% NaCl saline three times through the trachea. After lavage, the lung was ventilated and the chest opened. The lung was perfused with saline through the pulmonary artery and removed. The ovaries, fallopian tubes, and other organ tissues to be studied were removed from the carcass. Organ tissues of the nonpregnant rabbits were collected by similar procedures.

*To whom correspondence should be addressed at University of Wisconsin Perinatal Center, Meriter Hospital-Park, 202 South Park Street, Madison, WI 53715.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; PLBP, phospholipid-binding protein.

Copyright © 1995 by AOCS Press

Tissues of the same organ from two pregnant rabbits with the same gestational age or from two nonpregnant rabbits were pooled, washed with saline, and homogenized in two volumes of 0.01 M Tris-HCl, pH 7.4, containing 0.33 M sucrose using a tissue homogenizer (PCU Polytron; Drehzahlreglar, Kinematica, Switzerland). The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was further centrifuged at 100,000 × *g* for 60 min. The cytosolic fraction or the 100,000 × *g* supernatant was saved and stored at −20°C before use. The amniotic fluid was centrifuged at 200 × *g* for 5 min to remove debris, and the supernatant was reduced to about 1 mL using an ultrafiltration apparatus with an UM10 membrane and a Centricon™-10 (Amicon, Inc., Beaverly, MA). All sample preparations were carried out at 4°C or on ice. The amount of total proteins in each sample was determined using the Lowry method as described previously (11).

Western blotting, isoelectric focusing (IEF), and enzyme-linked immunosorbent assay (ELISA). Proteins in each sample were qualitatively analyzed by Western blotting and IEF, and quantitatively determined by ELISA using guinea pig antiserum raised against rabbit lung annexin I as previously described (11). The addition up to 80 ng of external annexin I into the samples of ovary and uterus showed a 76.3 ± 4.9% (n = 4) recovery. The ELISA results shown in this paper were expressed as μg annexin I/mg total sample protein from direct measurement without correction of the recovery. Annexins in the cytosolic proteins of the rabbit tissues also were immunoblotted against the mouse monoclonal antibodies to human annexins I, II, IV, and VI (Zymed Laboratories, Inc., San Francisco, CA) and the polyclonal antibodies to annexins I, II, III, IV, and V (kindly provided by Dr. Blake Pepinsky, Biogen, Cambridge, MA). The human fibroblast lysate proteins (Zymed Laboratories) were used as the positive control for the monoclonal antibodies. Human annexins I, III, IV, V, and bovine annexin II (kindly provided by Dr. Pepinsky) were used as positive control for the polyclonal anti-annexins antibodies as detailed elsewhere (11).

RESULTS

Annexins in reproductive organs. A 36 kDa PLBP was the only major cytosolic protein in the female rabbit reproductive organs that was immunorecognized by the guinea pig anti-rabbit lung annexin I antiserum (Fig. 1). Based on the same amounts of cytosolic proteins, the placenta, uterus, ovary, and fallopian tube appeared to have higher annexin I levels than most nonreproductive organ tissues, except the lung and the spleen (Fig. 1). The preimmunized serum of the same guinea pig prior to immunization was negative to the rabbit proteins. The 36 kDa protein in the uterus, ovary, fallopian tubes, and placenta had the same isoelectric point value as the lung annexin I (pI 6.0) (Fig. 2). The multiple bands of annexin I shown on the IEF gel probably resulted from charge modifications (11).

Monoclonal and polyclonal antibodies to human annexin I also reacted with the 36 kDa protein. Only in the uterus and placenta did a protein weakly react to monoclonal antibodies to annexin II. Anti-annexins III, IV, and V polyclonal antibodies, and anti-annexin IV and VI monoclonal antibodies did not react with any protein in the cytosolic fraction of the tissues we examined (data not shown). The 36 kDa protein, the annexin I, was by far the most prominent annexin in the female rabbit reproductive organs.

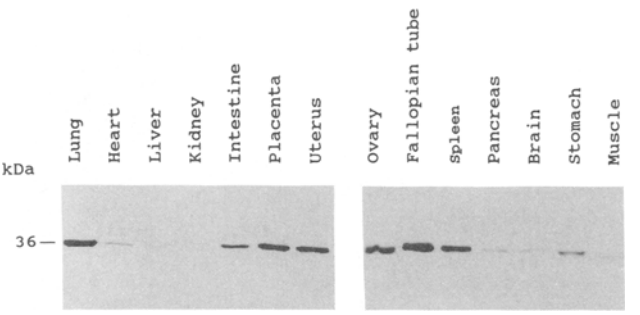


FIG. 1. Western blot of proteins in the cytosolic fraction of female rabbit reproductive organs against the anti-rabbit lung annexin I, a 36 kDa calcium-dependent phospholipid-binding protein, polyclonal antibody in comparison with proteins in nonreproductive tissues. Each sample applied to SDS polyacrylamide gel (7 × 8 cm) contained 10 μg cytosolic protein. Samples containing 200 μg proteins analyzed by 16 × 18 cm SDS gel yielded similar results. The amount of each reference phospholipid-binding protein purified from rabbit lung was 0.25 μg.

Pregnancy-related variation in annexin I in reproductive organs. The amount of annexin I in the ovary of 10-day- and 3-month-old nonpregnant rabbits was 56% lower than that in the ovary of the 5-month-old rabbits, 86% less than the 3-year-old rabbits, and 74% lower than the pregnant rabbits (Table 1). The New Zealand albino female rabbits reach maturity about five months after birth. Although age-related difference in annexin I in the uterus was not as obvious as in the ovary, the uterus of pregnant rabbits contained nearly twice

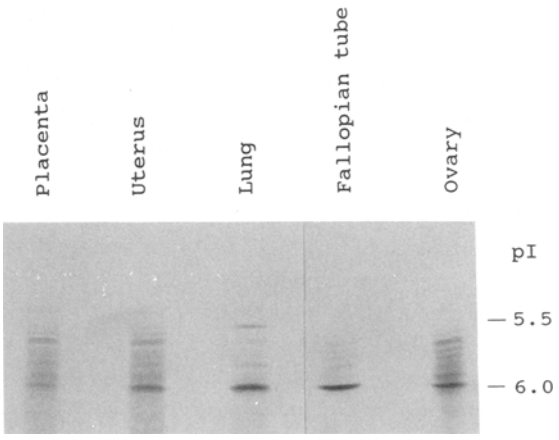


FIG. 2. Isoelectric focusing (IEF) and Western blot of cytosolic proteins (10 μg) in the female rabbit reproductive organs and the lung. The IEF agarose gel was 10 × 12.5 cm with a pH gradient of 3–10. IEF values of all specimens were identical.

TABLE 1
Annexin I Content in the Cytosolic Fractions of the Ovary, Uterus, and Placenta of Rabbits[a]

Age	Annexin I content (μg/mg protein)		
	Ovary	Uterus	Placenta
Nonpregnant rabbits			
10 d	5.6 ± 0.65	3.9 ± 0.25	—
3 mon	5.9 ± 0.34	3.1 ± 0.22	—
5 mon	9.0 ± 0.91	3.2 ± 0.06	—
3 yr	10.6 ± 0.20	4.2 ± 0.26	—
Pregnant rabbits (days of gestation)			
21	9.8 ± 0.78	5.6 ± 0.18	10.9 ± 0.31
26	10.8 ± 0.15	6.7 ± 0.36	15.8 ± 0.56
30	9.0 ± 0.63	8.0 ± 0.26	11.1 ± 0.89
31	10.2 ± 0.53	6.2 ± 0.06	10.1 ± 0.53

[a]The content of a 36 kDa calcium-dependent phospholipid-binding protein (annexin I) was determined by enzyme-linked immunosorbent assay as described in the text. The results are means ± SD of three tests.

the PLBP than that of nonpregnant rabbits (Table 1). The placenta contained higher annexin I than the ovary and the uterus, but the level remained relatively unchanged during pregnancy (Table 1).

Apparent hydrolysis and gestation-related increase in annexin I in amniotic fluid. Annexin I and its hydrolytic product, a 33 kDa protein, were detectable in amniotic fluid as early as at 21 days gestation (Fig. 3). However, on a per mg total protein basis, the amount of annexin in the amniotic fluid was less than that in the reproductive organs. Unlike in other organs where negligible breakdown products of annexin I could be detected, the amounts of annexin I and 33 kDa protein were about the same in the amniotic fluid, with an increase in quantity for both with advanced gestation. The volume of the amniotic fluid in the amniotic sac beyond the 27th gestational day was too small to be collected.

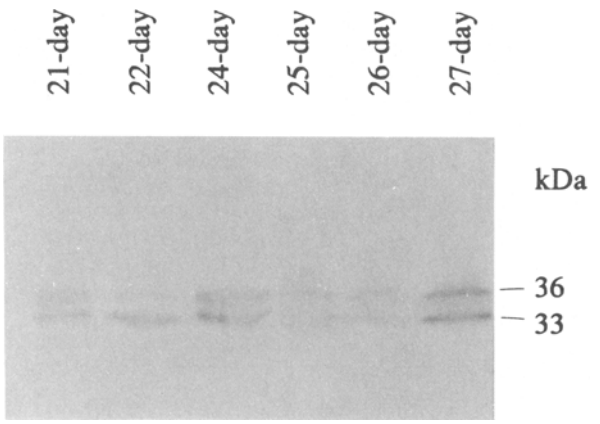


FIG. 3. Western blot of amniotic fluid against anti-rabbit lung annexin I antiserum. Each sample applied to sodium dodecyl sulfate gel (16 × 18 cm) contained 200 μg protein. Lanes of 21-day to 27-day represent gestational ages. Nearly equal amounts of annexin I (36 kDa) and its hydrolytic product (33 kDa) were present in the amniotic fluid, and the quantity of both annexin I and the breakdown product increased with increasing gestational days.

DISCUSSION

The polyclonal antibodies raised against a rabbit lung 36 kDa PLBP (15), previously identified as annexin I (11,16), together with monoclonal and polyclonal antibodies to other annexins, were employed to probe the expression and levels of annexins in the female rabbit reproductive organs, and in relation to maturity and pregnancy. With the polyclonal antibodies to rabbit lung annexin I, a rich presence of a 36 kDa protein was detected in the cytosolic fraction of all reproductive organs. The protein was also immunorecognized by monoclonal and polyclonal antibodies to human annexin I. There is little doubt that the immunoreacted 36 kDa protein in the reproductive organs is annexin I.

Annexin I was practically the sole annexin in the cytosolic fraction of the organs we examined, with a weak presence of annexin II in the uterus and placenta. Earlier, several investigators demonstrated multiple annexin proteins in a given tissue such as the lung and placenta (10,17,18). The absence of many of those annexins in our samples probably is related to the fact that the fractions used in our studies were prepared in mild buffer solutions. It is possible that other annexins associated with cellular membranes remained bound. In studies where multiple annexins were detected, the tissue was often treated harshly with buffers containing large quantities of detergents, chelating agents, and/or calcium ions.

The most interesting findings of the present study are the age- and pregnancy-related changes in annexin I levels in reproductive organs of the female rabbit. Annexin I in the ovary of immature rabbits was about one-half of that in the ovary of mature animals. Since annexin I is alleged to be involved in secretion and exocytosis, its increased levels may be needed to fulfill certain activities demanded by specific physiologic functions of the mature ovary, such functions may include ovualtion and hormone production. Similarly, annexin I in the uterus of pregnant rabbits was nearly twice as high as that of nonpregnant rabbits. It should not be difficult to imagine that activities normally carried out by uterine cells would be augmented and new activities assumed as the uterine wall is implanted with growing fetuses.

On a per mg protein basis, the placenta contained greater amounts of annexin I than other reproductive organs. The placenta is probably the most metabolically active tissue in sustaining a pregnancy, once fertilized eggs are implanted in the uterine wall and the fetuses are growing. The high annexin contents may simply reflect a heightened metabolic state of the tissue.

Annexin I in the amniotic fluid may be derived from multiple sources. It could be released from placental tissue as the protein is richly present in the placenta. Although cDNA does not predict a hydrophobic signal peptide on annexin molecules, the concept that annexin can be secreted has been suggested by Pepinsky *et al.* (19). Recent findings of Christmas

et al. (20) that annexin I is secreted by human prostate cells, probably *via* a previous unrecognized pathway, lend support to that notion. Conversely, annexin I in the amniotic fluid could originate from fetal tissue such as the lung. It has been shown that fetal lung surfactant components can appear in the amniotic fluid (21). This, together with our findings of the presence of annexin I in fetal rabbit lung and in the lung lavage fluid (11,22), suggests the possible release of annexin I from fetal lung into amniotic fluid. It is interesting to note that amniotic fluid had a relative high content of the 33 kDa protein. In the reproductive organs, as well as in fetal lung (11), the 33 kDa protein was barely or not detectable. The positive immunoreactivity of this protein to antibodies raised against annexin I is highly suggestive that the 33 kDa protein is a hydrolytic product of annexin I. It has been reported that annexin I can be subjected to limited proteolytic hydrolysis at the N-terminus to yield a 34 kDa peptide which can be recognized by the anti-annexin I polyclonal antibodies (23). If so, one must assume the presence of an annexin I hydrolytic enzyme in the amniotic fluid whose concentration would be considerably higher than in the organs. The pathophysiologic significance of the presence of the enzyme, and the function, if any, of the hydrolytic product remain to be delineated.

In conclusion, we have demonstrated a rich presence of annexin I in the reproductive organs of female rabbits, and the level of this protein increased in relation to maturity and/or pregnancy. Expression of annexins has been shown as a function of cellular growth state (24), and appears to be developmentally regulated in lymphocyte differentiation (25). Our finding of high concentrations of annexin I in the reproductive organs, and an increased level of this protein in mature animals and during pregnancy, is consistent with notion that this protein may play a regulatory role in the reproductive system of the female rabbit. The fact that annexin I is widely distriubuted in the reproductive organs may underscore particular activities (e.g., secretion and hormone production) common to these organs.

ACKNOWLEDGMENTS

This study was supported by the pulmonary SCOR grant 1P50 HL46478 and grant HL38744 from the National Institutes of Health. We are grateful to Dr. R. Blake Pepinsky of Biogen, Cambridge, MA, for kindly providing the annexins and anti-annexin antibodies.

REFERENCES

1. Romisch, J., and Paques, E.P. (1991) Annexins: Calcium-Binding Proteins of Multi-Functional Importance? *Med. Microbiol. Immunol. 180*, 109–126.
2. Creutz, C.E. (1992) The Annexins and Exocytosis, *Science 258*, 924–931.
3. Pollard, H.B., Burns, A.L., and Rojas, E. (1990) Synexin (annexin VII): A Cytosolic Calcium-Binding Protein Which Promotes Membrane Fusion and Forms Calcium Channels in Artificial Bilayer and Natural Membranes, *J. Membr. Biol. 117*, 101–112.
4. Flower, R. (1989) Lipocortin, *Biochem. Soc. Trans. 17*, 276–280.
5. Russo-Marie, F. (1991) Lipocortins: An Update, *Prostagland. Leuk. Essent. Fatty. 42*, 83–89.
6. Whitehouse, B.J. (1989) Lipocortins, Mediators of the Anti-Inflammatory Actions of Corticosteroids, *J. Endocrinol. 123*, 363–366.
7. Pepinsky, R.B., Tizard R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning, J.L., Chow, P., Burne, C., Huang, K.S., Pratt, D., Wachter, L., Hession, C., Frey, A.Z., and Wallner, B.P. (1988) Five Distinct Calcium and Phospholipid Binding Proteins Share Homology with Lipocortin I, *J. Biol. Chem. 263*, 10799–10811.
8. Melgunov, V.I. (1991) Annexins—A New Family of Ca^{2+}-Binding Proteins, *Biochemistry-USSR. 56*, 204–224.
9. Hauptmann, R., Maurer-Fogy, I., Krystek, E., Bodo, G., Andree, H., and Reutelingsperger,C.P.M. (1989) Vascular Anticoagulant β: A Novel Human Ca^{2+}/Phospholipid Binding Protein That Inhibits Coagulation and Phospholipase A_2 Activity. Its Molecular Cloning, Expression and Comparison with VAC–α, *Eur. J. Biochem. 185*, 63–71.
10. Pepinsky, R.B., and Hauptmann, R. (1992) Detection of VAC-β (annexin-8) in Human Placenta, *FEBS Lett. 306*, 85–89.
11. Tsao, F.H.C., Chen, X.R., Chen, X.M., and Vu, V.X. (1994) Immunocharacterization and Developmental Regulation of Rabbit Lung Calcium-Dependent Phospholipid-Binding Proteins, *Biochim. Biophys. Acta 1213*, 91–99.
12. Kaetzel, M.A., Hazarika, P., and Dedman, J.R. (1989) Differential Tissue Expression of Three 35-kDa Annexin Calcium-Dependent Phospholipid-Binding Proteins, *J. Biol. Chem. 264*, 14463–14470.
13. Lynch-Salamon, D.I., Everson, W.V., and Myatt, L. (1992) Decrease in Annexin I Messenger Ribonucleic Acid Expression in Human Amnion with Labor, *Am. J. Obstet. Gynecol. 167*, 1657–1663.
14. Myatt, L., Hirth, J., and Everson, W.V. (1992) Changes in Annexin (lipocortin) Content in Human Amnion and Chorion at Parturition, *J. Cell. Biochem. 50*, 363–373.
15. Tsao, F.H.C. (1990) Purification and Characterization of Two Rabbit Lung Ca^{2+}-Dependent Phospholipid-Binding Proteins, *Biochim. Biophys. Acta 1045*, 29–39.
16. Tsao, F.H.C., Hull, W.M., Strickland, M.S., Whitsett, J.A., Foo, T.K.F., Zografi, G., and DeLuca, P.M., Jr. (1991) Lung Calcium-Dependent Phospholipid-Binding Proteins: Structure and Function, *Biochim. Biophys. Acta 1081*, 141–150.
17. Khanna, N.C., Helwig, E.D., Ikebuchi, W., Fitzpatrick, S., Bajwa, R., and Waisman, D.M. (1990) Purification and Characterization of Annexin Proteins from Bovine Lung, *Biochemistry 29*, 4852–4862.
18. Romisch, J., and Heimburger, N. (1990) Purification and Characterization of Six Annexins from Human Placenta, *Biol. Chem. Hoppe-Seyler. 371*, 383–388.
19. Pepinsky, R.B., Sinclair, L.K., Browning, J.L., Mattaliano, R.J., Smart, J.E., Chow, E.P., Falbel, T., Ribolini, A., Garwin, J.L., and Wallner, B.P. (1986) Purification and Partial Sequence Analysis of a 37-kDa Protein That Inhibits Phospholipase A_2 Activity from Rat Peritoneal Exudates, *J. Biol. Chem. 261*, 4239–4246.
20. Christmas, P., Callaway, J., Fallon, J., Jones, J., and Haigler, H.T. (1991) Selective Secretion of Annexin 1, A Protein Without a Signal Sequence, by the Human Prostate Gland, *J. Biol. Chem. 266*, 2499–2507.
21. Hook, G.E.R., Gilmore, L.B., Tombropoulos, E.G., and Fabro, S.E. (1978) Fetal Lung Lamellar Bodies in Human Amniotic Fluid, *Am. Rev. Respir. Dis. 117*, 541–550.
22. Das, S.K., Chakrabarti, P., Tsao, F.H.C., Nayyar, T., and Mukherjee, S. (1992) Identification of Calcium-Dependent

Phospholipid-Binding Proteins (annexins) from Guinea Pig Alveolar Type II Cells, *Mol. Cell. Biochem. 115*, 79–84.
23. Ando, Y., Imamura, S., Hong, Y.M., Owada, M.K., Kakunaga, T., and Kannagi, R. (1989) Enhancement of Calcium Sensitivity of Lipocortin I in Phospholipid Binding Induced by Limited Proteolysis and Phosphorylation at the Amino Terminus as Analyzed by Phospholipid Affinity Column Chromatography, *J. Biol. Chem. 264*, 6948–6955.
24. Schlaepfer, D.D., and Haigler, H.T. (1990) Expression of Annexins as a Function of Cellular Growth State, *J. Cell Biol. 111*, 229–238.
25. Clark, D.M., Moss, S.E., Wright, N.A., and Crumpton, M.J. (1991) Expression of Annexin VI (p68, 67 kDa-calelectrin) in Normal Human Tissues: Evidence for Developmental Regulation in B- and T-Lymphocytes, *Histochemistry 96*, 405–412.

[Received June 15, 1994, in revised form March 22, 1995; Revision accepted March 23, 1995]

Inhibition of Cardiolipin Biosynthesis in the Hypoxic Rat Heart

Philip Cheng and Grant M. Hatch*

Departments of Internal Medicine and Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, R3E 3J7 Canada

ABSTRACT: Cardiolipin is the principal polyglycerophospholipid in the heart. The effect of hypoxia on cardiolipin biosynthesis was investigated in isolated rat hearts perfused in the Langendorff mode. Hearts were pulsed-labeled for 60 min with 0.1 mM [1,(3)-^{3}H]glycerol in Krebs Henseleit buffer saturated with either 95% O_2/5% CO_2 (control) or 95% N_2/5% CO_2 (hypoxic). Radioactivity incorporated into phosphatidylglycerol and cardiolipin were reduced 88% ($P < .05$) and 79% ($P < .05$), respectively, in hypoxic hearts compared to controls. In other experiments, hearts were pulse-labeled for 15 min with 1.4 mM [^{32}P]P_i in Krebs Henseleit buffer saturated with 95% O_2/5% CO_2 and subsequently perfused for 60 min under control or hypoxic conditions. The radioactivity incorporated into CDP–1,2-diacyl-*sn*-glycerol, phosphatidylglycerol, and cardiolipin were reduced 61% ($P < .05$), 71% ($P < .05$), and 70% ($P < .05$), respectively, in the hypoxic hearts compared to controls, indicating a decreased formation of CDP–1,2-diacyl-*sn*-glycerol in the hypoxic heart. The activities of the enzymes involved in cardiolipin biosynthesis and the cardiac pool sizes of cardiolipin, phosphatidylglycerol, and CDP–1,2-diacyl-1,2-diacyl-*sn*-glycerol were unaltered between hypoxic and control hearts. In contrast, cardiac adenosine-5′-triphosphate and CPT levels were decreased 94% ($P < .05$) and 92% ($P < .05$), respectively, in hypoxic hearts compared to controls. We postulate that the biosynthesis of the cardiac polyglycerophospholipid cardiolipin may be inhibited by a decreased adenosine-5′-triphosphate and cytidine-5′-triphosphate level in the heart.

Lipids 30, 513–519 (1995).

Polyglycerophospholipids are a special class of glycerolipids which include cardiolipin (CL), phosphatidylglycerol (PG), and *bis*(monoacylglycero)phosphate (1,2). Polyglycerophospholipids are widely distributed in nature and are found in plants, microorganisms, and animals (1). In the rat heart, PG was shown to comprise approximately 1.5% of the total phospholipid phosphorus mass whereas *bis*(monoacylglycero)-phosphate was not detected (3,4). CL is the principal polyglycerophospholipid in the heart muscle ranging from 9.0% in humans to 14.7% in rats of the total lipid phosphorus mass (1,3,4). CL is predominantly found within the inner leaflet of the inner mitochondrial membrane (5). CL aids in the association and function of mitochondrial membrane enzymes (6). Such enzymes include creatine kinase (7), cytochrome c oxidase (8), and the phosphate carrier (9). In addition, CL is essential for the proper functioning of the electron transport chain (10). Hence CL is a multifunctional phospholipid essential for cellular oxidative phosphorylation.

Mammalian mitochondria contain enzymes for the *de novo* synthesis of CL (11). In the rat heart, CL is synthesized *via* the CDP–1,2-diacyl-*sn*-glycerol (CDP–DG) pathway (3). The first step of the reaction is the conversion of phosphatidic acid (PA) to CDP–DG which requires CTP as substrate and is catalyzed by the enzyme CTP:PA cytidylyltransferase (12). The subsequent steps of PG biosynthesis involves the condensation of glycerol-3-phosphate with CDP–DG to form phosphatidylglycerolphosphate (PGP) *via* the action of PGP synthase and PGP phosphatase which rapidly dephosphorylates PGP to PG (12). Finally PG is converted to CL by a condensation reaction with CDP–DG catalyzed by CL synthase (13).

At present, the regulatory mechanisms that govern CL biosynthesis remain largely unknown, and regulation of these mechanisms in mammalian heart has received little attention. Myocardium derives most of its energy from mitochondrial oxidative phosphorylation under normal physiological conditions (14). When available oxygen is limited, as in the case of myocardial hypoxia, high-energy phosphate stores decline (15). The effect of a reduction in cardiac high-energy stores on CL biosynthesis in mammalian heart was unknown. Thus, the purpose of this study was to determine the effect of hypoxia on CL biosynthesis in the isolated perfused rat heart. In this study, we demonstrate that CL biosynthesis is decreased when the heart is perfused under hypoxic conditions.

MATERIALS AND METHODS

Materials. [1,(3)-^{3}H]Glycerol, [^{32}P]P_i, and [U-^{14}C]glycerol-3-phosphate were obtained from Amersham (Oakville, Ontario, Canada). Thin-layer plates (silica gel 60, 0.25 mm thickness) and Ecolite™ scintillation cocktail were obtained from CanLab Division of Baxter Co. (Winnipeg, Manitoba, Canada). All other biochemical reagents were of analytical grade and were obtained from either Fisher Scientific (Ed-

*To whom correspondence should be addressed at Department of Physiology, Basic Medical Sciences Building Rm. 440, Faculty of Medicine, University of Manitoba, 730 William Ave., Winnipeg, Manitoba, R3E 3J7 Canada.

Abbreviations: CDP–DG, CDP–1,2-diacyl-*sn*-glycerol; CL, cardiolipin; KHB, Krebs Henseleit buffer; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; TBA, tetrabutylammonium.

Copyright © 1995 by AOCS Press

monton, Alberta, Canada); Sigma Chemical Co. (St. Louis, MO); or CanLab Division of Baxter Co. Male Sprague-Dawley rats (100–220 g) were used for all the experiments.

Perfusion of isolated rat hearts in the Langendorff mode with [1,(3)-^{3}H]glycerol or [^{32}P]P_i. Male Sprague-Dawley rats were sacrificed by decapitation. The heart was quickly removed and cannulated *via* the aorta using a modified syringe needle (18 gauge). Then 5 mL of Krebs Henseleit buffer (KHB) (16) was injected through the coronary circulation with a 5-cc syringe to remove the remaining blood. Immediately after, the heart was placed on the perfusion apparatus (Fig. 1) and perfused in the Langendorff mode (17) with 12.5 mL KHB containing 0.1 mM [1,(3)-^{3}H]glycerol (2.5 μCi/mL) for 60 min in continuous pulse-labeling experiments. The concentration of glycerol (0.1 mM) used throughout this experiment was representative of the physiological plasma concentration in rats (18). The perfusate was saturated either with 95% O_2/5% CO_2 (control) or 95% N_2/5% CO_2 (hypoxic). The pO_2 of the O_2 saturated buffer was 69.6 ± 2.0 kPa, while the pO_2 for the N_2 saturated buffer was 2.9 ± 0.7 kPa (19). In the pulse-chase experiments, the heart was perfused for 15 min with KHB containing 1.4 mM [^{32}P]P_i (67 μCi/mL) under control condition and subsequently chased with KHB (1.4 mM P_i) for another 60 min under either control or hypoxic conditions.

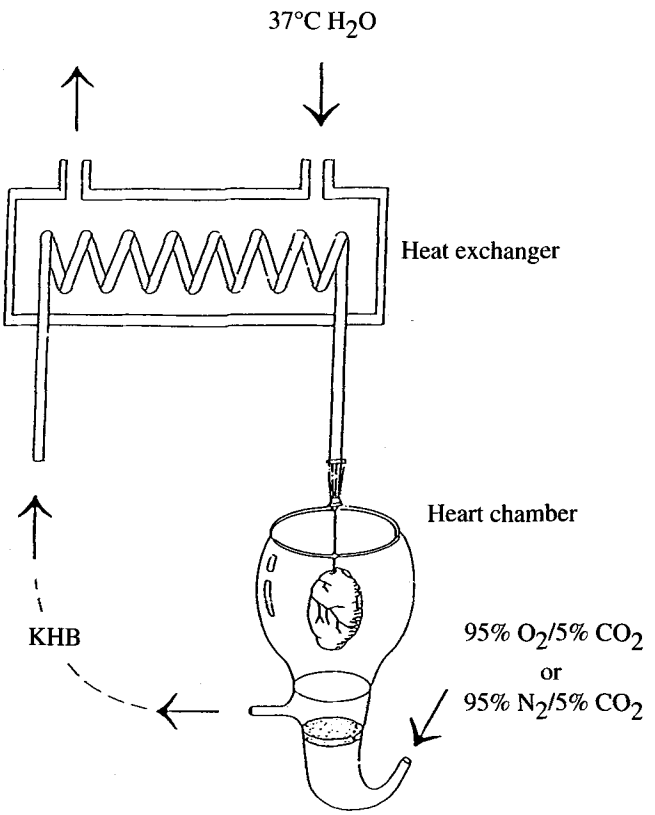


FIG. 1. Retrograde perfusion apparatus. Krebs Henseleit buffer (KHB) flows through a heat exchanger, is warmed to 37°C, and subsequently flows into the heart *via* the aorta in a retrograde manner. The buffer is collected in the heart chamber and recirculated. The pool of buffer in the heart chamber is saturated with either 95% oxygen/5% carbon dioxide (control) or 95% nitrogen/5% carbon dioxide (hypoxic).

Extraction and isolation of radioactive phospholipids. Subsequent to perfusion, 10 mL of air was injected into the heart to remove perfusate that remained in the coronary circulation, and the heart was blotted dry. The heart was weighed (typically 0.30–0.50 g) and homogenized by a 20 s burst of a Polytron® Homogenizer (Kinematika, Lucerne, Switzerland) in 5 mL of chloroform/methanol (2:1, vol/vol). Tissue left on the homogenizer probe was removed by homogenization with another 5 mL of chloroform/methanol (2:1, vol/vol). The two 5-mL homogenates were combined and centrifuged at full speed for 10 min in a bench-top centrifuge (Model TJ-6; Beckman Instruments, Mississauga, Ontario, Canada) to pellet debris. After centrifugation, the homogenate was decanted into a 16 × 125 mm screw-cap tube. Addition of 5 mL of 0.73% NaCl to the homogenate resulted in the formation of organic and aqueous phases. The tube was vortexed and centrifuged at full speed for 10 min in a bench-top centrifuge, causing a biphasic separation. The upper aqueous phase was removed by aspiration, and the remaining organic layer was further washed with 5 mL of theoretical upper phase (chloroform/methanol/0.9% NaCl, 3:48:47, by vol). The tube was vortexed and centrifuged as described above. The resulting upper aqueous phase and interphase were removed, and the remaining organic phase was dried down under a stream of N_2 gas. The dried organic lipid sample was resuspended in 100 μL chloroform/methanol (2:1, vol/vol). Thin-layer chromatography plates treated with 0.4 M boric acid were used to resolve individual phospholipid species (4). Before application of the lipid sample, the thin-layer plate was heat activated for one hour at 145°C. The thin-layer plate was allowed to cool; then 25 μL of the resuspended lipid sample was placed on the plate. The thin-layer plate was subjected to a two-dimensional separation. In the first dimension, the plate was developed in a solvent system containing chloroform/methanol/NH_4OH/water (70:30:2:3, by vol). The second dimension, at 90° to the first separation, was performed in a solvent system containing chloroform/methanol/water (65:35:5, by vol). The thin-layer plate was allowed to dry overnight, and the separated phospholipids were visualized by exposing the plate to iodine vapor. Corresponding radioactive phospholipids of interest were removed and placed in 6-mL scintillation vials. Radioactivity of [^{32}P] and [^{3}H] incorporated phospholipids were determined immediately and 48 h, respectively, after the addition of 5 mL of the Ecolite scintillation cocktail. The radioactivity was determined in a Beckman Model LS 3801 liquid scintillation counter. CDP–DG was resolved by a separation system containing chloroform/methanol/acetic acid/water (50:24:4:8, by vol) (20). A 10-μL sample of the organic phase was placed on a thin-layer plate with 25 μL of 2 mM CDP–DG standard. The CDP–DG band was identified by exposing the thin-layer plate to iodine vapor, and the corresponding silica gel was removed and placed into a 6-mL scintillation vial and radioactivity determined as described above.

Determination of CL, PG, and CDP–DG pool sizes. In phospholipid mass determination experiments, 35 μL of the organic phase was placed on borate-treated thin-layer plates and developed in the two-dimensional chromatography system described above (4). Silica gel corresponding to the phospholipids was identified by exposure to iodine vapor and were removed from the plate and placed in 13 × 100-mm test tubes. The phosphorus content of the phospholipid was determined *via* the method of Rouser *et al.* (21). The separation of CDP–DG from a 15 μL aliquot of the organic phase, without standard CDP–DG, was performed as described above (20). The phosphorus content was determined as described above and confirmed by the more sensitive malachite green phosphorus determination method (22).

Preparation of subcellular fractions. Crude mitochondrial fractions were prepared for assay of enzyme activities of the following enzymes: CTP:PA cytidylyltransferase, PGP synthase, PGP phosphatase, and CL synthase. The animal was sacrificed by decapitation, and the heart was quickly removed and perfused with KHB saturated with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 for 60 min as previously described. Subsequent to perfusion, a 10% homogenate was prepared by homogenizing the heart (Polytron® 20-s burst) in homogenizing buffer containing 5 mM Tris-HCl, 0.25 M sucrose, 0.145 M NaCl, pH 7.4. For CL synthase assays, the heart was homogenized in 10 mM Tris-HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4. The homogenate was then centrifuged for 10 min at 1,000 × *g* (Sorvall RC-5 Superspeed refrigerated centrifuge with SS-34 rotor; Dupont, Mississauga, Ontario, Canada). The resulting pellet was discarded, and the supernatant was centrifuged at 12,000 × *g* for 15 min. The resulting pellet was resuspended in homogenizing buffer (0.5–1.0 mL) using a Dounce tissue grinder (15 hand strokes) and was designated the mitochondrial fraction. A 5-μL aliquot of the crude mitochondrial fraction was taken to determine the protein concentration.

Assay of enzyme activities involved in CL biosynthesis. Enzyme activities were determined in the mitochondrial fraction prepared from the rat hearts after 60 min of perfusion under control or hypoxic conditions. CTP:PA cytidylyltransferase was assayed by determining the conversion of [5-^{3}H]CTP to [5-^{3}H]CDP–DG (3). Test tubes (16 × 100 mm) treated with dimethyldichlorosilane 2% in 1,1,1-trichloroethane were dried overnight at 142°C and were used as the reaction tubes. The reaction mixture contained 50 mM Tris-maleate, pH 6.5, 10 mM [5-^{3}H]CTP (specific activity, 5,739 dpm/nmol), 0.1 mg of mitochondrial protein, 0.5 mM PA dissolved in 0.15 M Triton X-100. Homogenizing buffer was added to yield a final volume of 90 μL. The reaction was initiated by the addition of 10 μL of 0.2 M $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30°C for 5 min, and the reaction was terminated by the addition of 0.5 mL of 0.1 M HCl in methanol. To the mixture was added 1 mL of chloroform and 1.5 mL of 1.0 M $MgCl_2$. The suspension was vortexed, and the mixture was centrifuged at full speed for 10 min in a bench-top centrifuge. The aqueous phase was removed by suction, and 500 μL of the organic phase was placed in a 6-mL scintillation vial. The organic phase in the vial was evaporated to dryness at 60°C in a water bath. Then 5 mL of scintillation cocktail was added to the vial and the radioactivity determined.

PGP synthase activity was assayed by determining the conversion of [U-^{14}C]glycerol-3-phosphate to phosphatidyl[U-^{14}C]glycerol-3-phosphate (23). The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 10 mM ß-mercaptoethanol (prepared fresh), 0.5 mM [U-^{14}C]glycerol-3-phosphate (specific activity, 11,794 dpm/nmol), 2 mM CDP–DG (dipalmitoyl) dissolved 10 mM Triton X-100, and 50–100 μg of mitochondrial protein to a final volume of 90 μL. The reaction mixture was initiated by the addition of 10 μL of 0.15 M $MgCl_2 \cdot 6H_2O$. The mixture then was incubated at 30°C for 10 min. Next, 0.5 mL of 0.1 M HCl in methanol was added to terminate the reaction. To the mixture was added 1 mL of chloroform and 1.5 mL of 1 M $MgCl_2$. Radioactivity in the organic fraction was determined as described for CTP:PA cytidylyltransferase.

PGP phosphatase activity was assayed by determining the conversion of phosphatidyl[U-^{14}C]glycerol-3-phosphate to phosphatidyl[U-^{14}C]glycerol (24). The reaction mixture contained 50 mM Tris-maleate, pH 6.5, 10 mM β-mercaptoethanol, 0.5 μM phosphatidyl[^{14}C]glycerol-3-phosphate (specific activity, 15,000 dpm/nmol), and 0.1 μg of mitochondrial protein to a final volume of 90 μL. The reaction mixture was incubated at 37°C for 5 min and was terminated by addition of 2 mL of chloroform/methanol/HCL (100:100:0.6, by vol). This was followed by the addition of 1 mL of chloroform and 1.5 mL of 0.73% NaCl to cause biphasic separation. The tube was vortexed and centrifuged at full speed for 10 min in a bench-top centrifuge. The resulting aqueous phase was removed, and the organic phase was dried under N_2 gas. The residue was resuspended in 25 μL of chloroform/methanol (2:1, vol/vol), and 20 μL was placed onto a 0.5 N oxalate-treated thin-layer plate (25). Oxalate plates were prepared by immersing the thin-layer plate in a solution of 0.5 N oxalic acid, and they were dried overnight before use. This treatment is to ensure clear separation of PGP and PG in the thin-layer plate. Standard PG (3 μL) was placed on the thin-layer plate with the sample. The solvent system used for development of the thin-layer plate contained chloroform/methanol/HCl (87:13:0.2, by vol). Iodine vapor was used to visualize the separated phospholipids. The silica gel representing PG was removed and the radioactivity determined.

CL synthase activity was assayed by determining the conversion of phosphatidyl[U-^{14}C]glycerol to [^{14}C]CL (26). The reaction mixture contained 0.3 mM EDTA dissolved in 45 mM Tris-HCl, pH 8.5, 50 μM CDP–DG, 8 μM phosphatidyl[U-^{14}C]glycerol (specific activity, 45,000 dpm/nmol), and 0.1 mg of mitochondrial protein to a final volume of 90 μL. The reaction was initiated by the addition of 5 mM $CoCl_2 \cdot H_2O$ and incubated at 37°C for 120 min. The reaction was terminated by the addition of 3 mL of chloroform/methanol (2:1, vol/vol). This mixture was then decanted into a screw-cap tube (16 × 125 mm), and 1 mL of chloroform followed

by 1 mL of water were added and immediately mixed. The mixture was centrifuged at full speed in a bench-top centrifuge for 10 min. The upper aqueous phase was removed by suction, and the organic phase was evaporated to dryness under N_2 gas. The residue lipid sample was resuspended in 25 μL of chloroform/methanol (2:1, vol/vol), and 20 μL was placed onto a 0.5 N oxalate-treated thin-layer plate. Standard PG (3 μL) and CL (2 μL) were placed on the thin-layer plate with the sample. The same solvent system used for isolation of PG in the PGP phosphatase assay was employed. Iodine vapor was used to visualize the separated phospholipids, and silica gel representing CL was removed and the radioactivity was determined.

Determination of ATP and CTP level. Subsequent to perfusion under either control or hypoxic conditions, the heart was weighed and homogenized in 5 mL chloroform/methanol (2:1, vol/vol). Tissue left on the homogenizer probe was removed by another 5 mL of chloroform/methanol. The two 5-mL homogenates were combined and centrifuged at full speed for 10 min in a bench-top centrifuge to pellet debris. After centrifugation, the homogenate was decanted into a 16 × 125-mm screw-cap tube. Addition of 3 mL of water to the homogenate resulted in the formation of organic and aqueous phases. The tube was vortexed and centrifuged at full speed for 10 min in a bench-top centrifuge. The resulting upper aqueous phase was collected in a 16 × 125 mm test tube. The organic phase was washed twice with 3 mL of water and the aqueous phase pooled. The pooled aqueous phase was dried down under air (24–48 h), and the residue was resuspended in 200 μL of water. The sample was diluted either 20 times (for ATP determination) or 5 times (for CTP determination) with tetrabutylammonium (TBA) buffer. TBA buffer contained 0.03 M KH_2PO_4, 0.02 M TBA phosphate in 19% acetonitrile, pH adjusted to 2.65 using H_3PO_4. Next, 20 μL of the diluted sample was injected into an Ultrasphere-ODS C_{18} reversed-phase high-performance liquid chromatography column for the detection of either ATP or CTP. In an isocratic system, TBA was used to elute nucleotide from the column. The flow rate was set at 2 mL/min and 1.2 mL/min for ATP and CTP, respectively. Nucleotides were detected by an ISCO® UA-5 Absorbance/Fluorescence Detector (Beckman Instruments). The numerical data was generated by a Beckman 450 Data System/Controller.

Other analytical procedures. Protein concentration was determined by the method of Bradford (27). Student's *t*-test was used for the determination of statistical significance. The level of significance was defined as $P < 0.05$.

RESULTS

Effect of hypoxia on CL biosynthesis. To determine if the biosynthesis of CL was effected by hypoxia, rat hearts were perfused in the Langendorff mode with 0.1 mM [1,(3)-^{3}H]glycerol (2.5 μCi/mL) for 60 min in a continuous pulse-labeling experiment under control or hypoxic conditions and the radioactivity incorporation into PG and CL in the organic fraction was determined. Total radioactivity incorporated into the heart was $6.1 \pm 0.7 \times 10^6$ dpm/g heart in the control hearts and $6.4 \pm 0.2 \times 10^6$ dpm/g heart in the hypoxic hearts. Radioactivity incorporated into the organic fraction was reduced 78% ($P < .05$) from $1.8 \pm 0.5 \times 10^6$ dpm/g heart in control hearts to $0.4 \pm 0.1 \times 10^6$ dpm/g heart in the hypoxic hearts. Radioactivity incorporated into CL and PG were significantly reduced, 79% ($P < .05$) and 88% ($P < .05$), respectively, in the hypoxic hearts compared to controls (Fig. 2). Thus, hypoxia caused an apparent reduction in biosynthesis of PG and CL. A problem with continuous pulse-labeling experiments is that changes in the radioactivity incorporated into metabolites may be due to isotope dilution or altered uptake of radioactive precursors (28). Therefore, a pulse-chase protocol was designed to circumvent this problem. Rat hearts were pulse-labeled for 15 min with 1.4 mM [^{32}P]P_i (67 μCi/mL) in 95% O_2-saturated KHB and subsequently the radioactivity was chased with nonradioactive KHB (1.4 mM) for 60 min under control or hypoxic conditions. The radioactivity in both control and hypoxic hearts was $3.1 \pm 0.5 \times 10^6$ dpm/g heart at the beginning of the chase. The radioactivity incorporated into CDP–DG, PG, and CL were determined at time 0 and 60 min of the chase. Radioactivity incorporated into CDP–DG, PG, and CL was increased with the time of perfusion in both

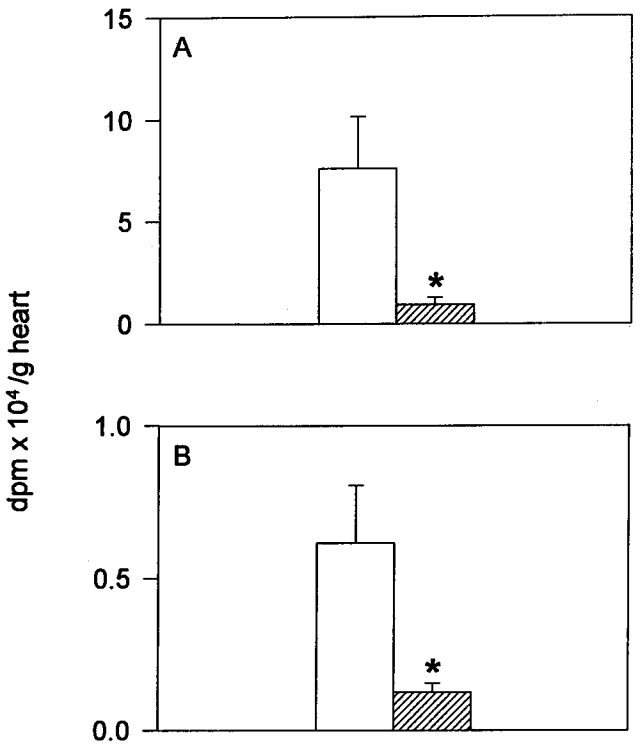


FIG. 2. Radioactivity incorporated into cardiolipin (CL) and phosphatidylglycerol (PG) in hearts perfused with 0.1 mM [1,(3)-^{3}H]glycerol. Rat hearts were perfused for 60 min with Krebs Henseleit buffer containing 0.1 mM [1,(3)-^{3}H]glycerol under control (open columns) or hypoxic (hatched columns) conditions. The hearts were homogenized, and PG and CL were isolated by thin-layer chromatography and the radioactivity associated with PG (A) and CL (B) determined. Vertical bars represent the mean ± SD of at least three hearts (*$P < .05$).

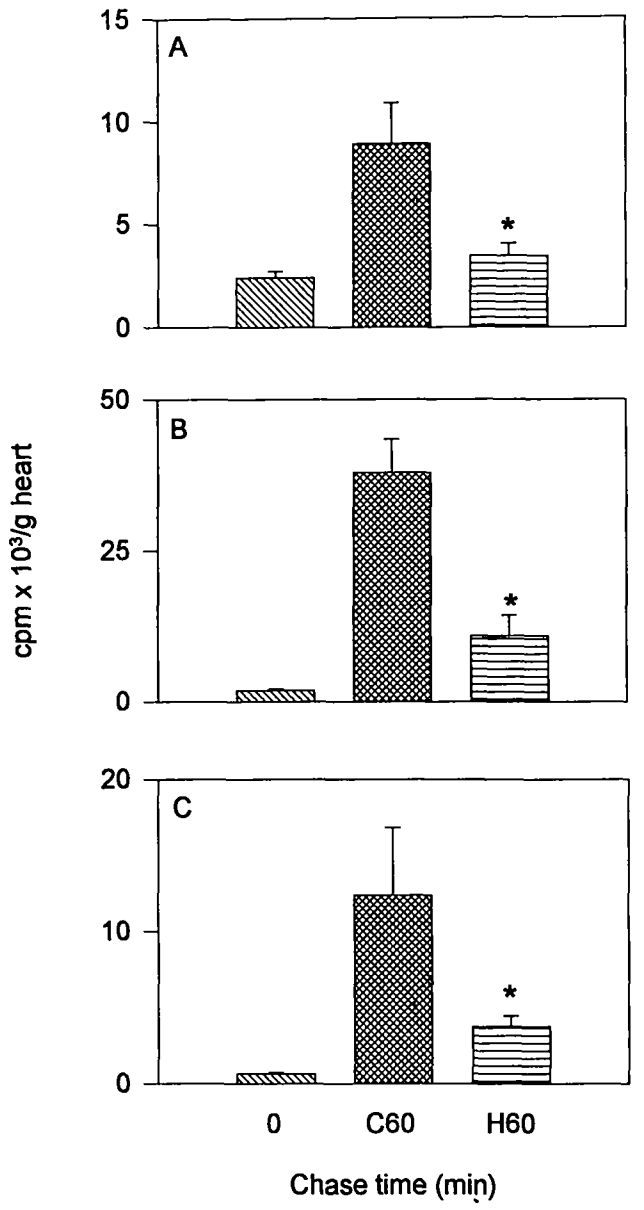


FIG. 3. Pulse-chase experiment of 1.4 mM [^{32}P]P_i incorporated into CDP-1,2-diacyl-*sn*-glycerol (CDP–DG), PG, and CL. Rat hearts were pulsed for 15 min with KHB containing 1.4 mM [^{32}P]P_i under control conditions (0). Subsequently, the hearts were perfused for 60 min with Krebs Henseleit buffer under control (C60) or hypoxic (H60) conditions. The hearts were homogenized subsequent to perfusion, and various phospholipids were isolated by thin-layer chromatography. The radioactivity associated with (A) CDP–DG, (B) PG, and (C) CL was determined. Vertical bars represent the mean ± SD of three hearts (*$P < .05$). See Figure 2 for abbreviations.

control and hypoxic hearts (Fig. 3). In contrast, radioactivity incorporated into CDP–DG, PG, and CL was decreased 61% ($P < .05$), 71% ($P < .05$), and 70% ($P < .05$), respectively, in hypoxic hearts compared to controls (Fig. 3). These experiments suggested that a reduction in the formation of new CDP–DG, PG, and CL occurred in the hypoxic heart.

Effect of hypoxia on the pool size of CL, PG, and CDP–DG. To determine if hypoxia affected CL mass, rat hearts were perfused under control or hypoxic conditions for 60 min. Subsequently, the phospholipids were extracted after 60 min of perfusion and the phospholipid phosphorus determined. As seen in Figure 4, there were no significant changes in the pool sizes of CL, PG, and CDP–DG between hypoxic hearts and controls. The phosphorus mass of other phospholipids were determined from the heart extract and were unaltered. PA, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin, and lysophosphatidylcholine content were 38 ± 24, 1,077 ± 103, 6,858 ± 138, 7,946 ± 958, 655 ± 72, 177 ± 31, and 144 ± 19 nmol/g, respectively, of heart in control hearts and were unchanged after perfusion under hypoxic conditions. Thus, the

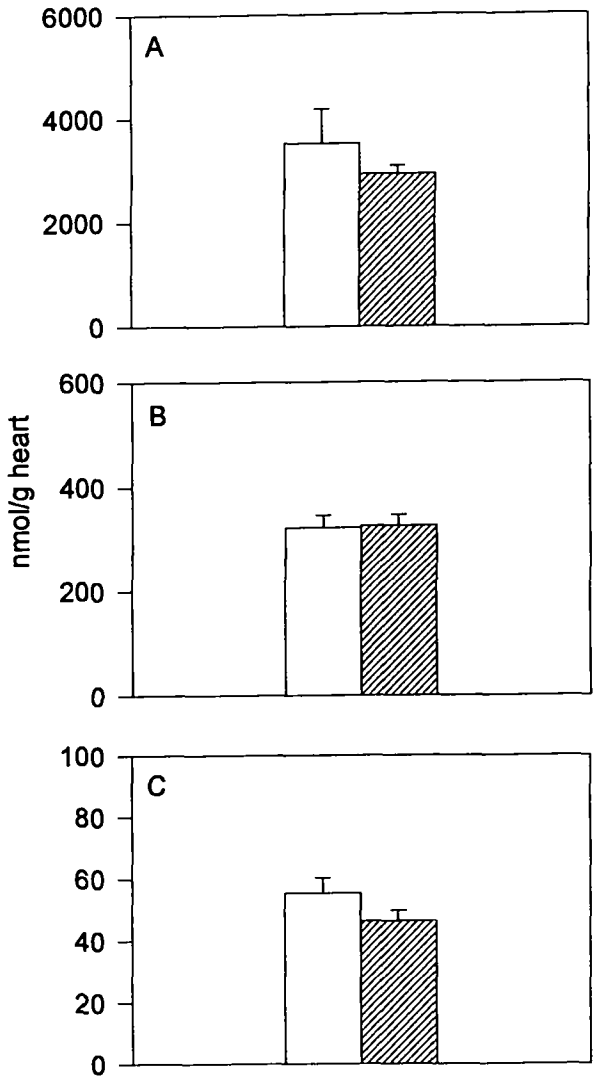


FIG. 4. Pool size of CL, PG, and CDP–DG in control and hypoxic hearts. Hearts were perfused for 60 min with KHB under control (open columns) or hypoxic (hatched columns) conditions. The hearts were homogenized subsequent to perfusion and phospholipids were isolated by thin-layer chromatography. The pool sizes were determined for: (A) CL, (B) PG, (C) CDP–DG. The vertical bars represent the mean ± SD of at least three hearts.

TABLE 1
***In vitro* Enzyme Activities Involved in Cardiolipin Biosynthesis[a]**

Enzyme	Activity (pmol/min per mg of protein) Control	Hypoxic
CTP:PA cytidylyltransferase	33.1 ± 6.5 (3)	33.5 ± 9.7 (3)
PGP synthase	72.6 ± 9.7 (3)	71.7 ± 13.5 (3)
CL synthase	1.0 ± 0.7 (4)	1.1 ± 0.5 (3)
PGP phosphatase	0.70 ± 0.05 (3)[b]	0.72 ± 0.13 (3)[b]

[a]Rat hearts were perfused for 60 min with Krebs Henseleit buffer under either control or hypoxic conditions. Subsequent to perfusion, the hearts were homogenized, and the activities of the enzymes involved in cardiolipin (CL) biosynthesis were determined in isolated mitochondrial fractions prepared from the homogenate. The enzymes were assayed under optimal conditions. Values represent the mean ± SD (number of hearts). PGP, phosphatidylglycerolphosphate; PA, phosphatidic acid.
[b]nmol/min per mg of protein.

reduction of radioactivity in CL in the hypoxic heart was not caused by a change in the pool size of CL or its precursors.

Effect of hypoxia on the in vitro *enzyme activities involved in CL biosynthesis.* The decrease in radioactivity observed in CL could be due to a change in the activity of one of the enzymes involved in CL biosynthesis. Thus, the specific activities of the enzymes involved in CL biosynthesis from control and hypoxic hearts were determined. Hearts were perfused for 60 min under control or hypoxic conditions. Subsequent to perfusion, the hearts were homogenized, and the mitochondrial fraction was prepared by differential centrifugation. CTP:PA cytidylyltransferase, PGP synthase, PGP phosphatase, and CL synthase were assayed in the isolated mitochondrial fractions. Enzyme activities between the control and hypoxic hearts were unaltered (Table 1). Thus, the reduction of radioactivity in CL in the hypoxic heart was not caused by a change in the activity of the enzymes involved in CL biosynthesis.

Effect of hypoxia on ATP and CTP level of the heart. In the CDP–DG pathway, CTP is a cofactor required for the conversion of PA to CDP–DG. Since the continuous-pulse and pulse-chase labeling studies indicated a possible decreased formation of new CDP–DG, the levels of cardiac ATP and CTP were determined in the hearts perfused for 60 min under control or hypoxic conditions. It was found that in the hypoxic hearts, ATP and CTP levels were significantly decreased 94 and 92%, respectively, when compared to controls (Table 2). Thus, the large reduction of cardiac CTP may have been responsible for the decreased formation of CDP–DG in the hypoxic hearts and the resulting decrease in radioactivity incorporated into CL.

TABLE 2
ATP and CTP Concentration in Control and Hypoxic Hearts[a]

	ATP	CTP
	(nmol/ g heart)	
Control	633 ± 110 (3)	5.25 ± 0.85 (3)
Hypoxic	39 ± 8 (3)[b]	0.41 ± 0.06 (3)[b]

[a]Rat hearts were perfused for 60 min with Krebs Henseleit buffer saturated with either control or hypoxic conditions. Subsequent to perfusion ATP and CTP levels were quantified by C_{18} reverse-phase high-performance liquid chromatography. Values represent the mean ± SD (number of hearts).
[b]$P < 0.05$.

DISCUSSION

The purpose of this study was to determine if hypoxia affected CL biosynthesis in the heart. In order to examine this hypothesis, we perfused hearts in the Langendorff mode with radioactive glycerol or P_i under control or hypoxic conditions. Langendorff perfusion is a highly reproducible, stable method which is especially useful in metabolic labeling studies (29). In the continuous pulse-labeling studies, we observed an apparent reduction in the biosynthesis of new PG and CL. In addition, the pulse-chase studies indicated that the radioactivity incorporated into CDP–DG, PG, and CL was reduced in the hypoxic hearts compared to controls at 60 min of perfusion. Thus, perfusion of the heart under hypoxic conditions inhibits CL biosynthesis.

The level of cardiac ATP and CTP was reduced by perfusing the isolated rat heart under hypoxic conditions consistent with that of hamster hearts perfused under similar conditions (19). Indeed, following a 60-min perfusion under hypoxic conditions, we observed a 94% decrease in the cardiac ATP level compared to controls. ATP is required for the amination reaction in CTP production (30); therefore, loss of ATP would consequently reduce the biosynthesis of CTP. We observed a corresponding reduction of 92% in the cardiac CTP level in hypoxic hearts compared to controls.

The mechanism of the reduction of CL biosynthesis under hypoxic conditions was investigated. Our results indicated that the activities of the enzymes of the CDP–DG pathway were unaltered between the hypoxic and control hearts. This is in contrast to previous studies on PC biosynthesis in the hamster heart (19,31). In those studies, CTP:phosphocholine cytidylyltransferase activity was increased as a compensatory mechanism for the reduction in cardiac CTP level. The results of the present study suggest that there may not be such a compensatory mechanism for reduced CL biosynthesis caused by lowered cardiac CTP.

ATP is required to convert glycerol to glycerol-3-phosphate, and oxygen is required for oxidative phosphorylation and the conversion of [^{32}P]Pi to [^{32}P]ATP. Thus, the reduction in labeling of PG and CL in the [^{32}P]Pi perfused hearts was not surprising. Depletion of cardiac ATP and, hence, CTP might also result in a decreased formation of new CDP–DG, producing less available radioactive CDP–DG to form CL. Indeed, this was observed in the pulse-chase studies. Despite the drastic reduction in the ATP and CTP level of the heart, the pool sizes of CDP–DG, PG, and CL in the hypoxic hearts were unchanged compared to controls. One possible explanation for this is that a large decrease of phospholipid mass would simply be incompatible with the viability of the heart. Alternatively, the total phospholipid pool may eventually decrease but was not detectable after 60 min of perfusion under

hypoxic conditions. Perhaps an alteration in phospholipid mass would be apparent if the hypoxic treatment was prolonged. However perfusion for periods beyond 60 min under hypoxic conditions causes irreversible damage to the heart (19).

Previous studies have set a precedent for the regulation of CL biosynthesis under conditions of altered mitochondrial oxidative metabolism. For example, thyroid hormone treatment of rats increased the oxidative activity of heart mitochondria and increased the CL content in these hearts 51% (32). In rat liver, CL synthase activity was increased 52% in rats treated with thyroxine (33). Such an increase in CL synthase activity has offered a plausible mechanism for the increased CL observed in the tissues of thyroid hormone-treated animals. The above studies are in contrast to our study utilizing acute hypoxia, since thyroid-induced increased oxidative activity appears to be associated with increased CL biosynthesis. The observed reduction in cardiac PG and CL biosynthesis in hearts perfused under hypoxic conditions is interesting in light of the observation that ultraviolet A radiation inhibits PG and CL biosynthesis in N.C.T.C. 2544 human keratinocytes (34) and decreased ATP levels have been observed in lymphocytes exposed to a ultraviolet A radiation (35).

In summary, we have utilized an intact isolated rat heart to study the effect of hypoxia on CL biosynthesis. We have shown that perfusion of the heart under hypoxic conditions will result in the reduction of newly synthesized CL. The results provide strong evidence to support that the biosynthesis of CL is governed by the ATP and CTP level of the heart.

ACKNOWLEDGMENTS

This study was supported by a grant from the Heart and Stroke Foundation of Manitoba. The authors would like to thank Dr. S.G. Cao for helpful discussions and technical assistance. Illustration of retrograde perfusion apparatus by O.K.H. Cheng was greatly appreciated. P.C. is the recipient of a Department of Internal Medicine graduate studentship. G.M.H. is a Heart and Stroke Foundation of Canada Scholar.

REFERENCES

1. Hostetler, K.Y. (1982) in *Phospholipids* (Hawthorne, J.N., and Ansell, G.B., eds.), pp. 215–261, Elsevier Biomedical Press, Amsterdam.
2. Vance, D.E. (1985) in *Biochemistry of Lipids and Membranes* (Vance, D.E., and Vance, J.E., eds.), pp. 263–265, The Benjamin Cummings Publishing Company, Menlo Park, California.
3. Hatch, G.M. (1994) *Biochem. J. 297*, 201-208.
4. Poorthuis, B.J.H.M., Yazaki, P.J., and Hostetler, K.Y. (1976) *J. Lipid Res. 17*, 433–437.
5. Krebs, J.J.R., Hauser, H., and Carafoli, E. (1979) *J. Biol. Chem. 254*, 5308–5316.
6. Bell, R.M., and Coleman, R.A. (1980) *Ann. Rev. Biochem. 49*, 459–487.
7. Cheneval, D., and Carafoli, E. (1988) *Eur. J. Biochem. 171*, 1–9.
8. Robinson, N.C. (1993) *J. Bioenerg. Biomembr. 25*, 153–161.
9. Mende, P., Huther, F.J., and Kadenbach, B. (1983) *FEBS Lett. 158*, 331–334.
10. Ohtsuka, T., Nishijima, M., Suzuki, K., and Akamatsu, Y. (1993) *J. Biol. Chem. 268*, 22914–22919.
11. Schlame, M., and Haldar, D. (1993) *J. Biol. Chem. 268*, 74–79.
12. Kiyasu, J.Y., Pieringer, R.A., Paulus, H., and Kennedy, E.P. (1963) *J. Biol. Chem. 238*, 2293–2298.
13. Hostetler, K.Y., Van Den Bosch, H., and Van Deenen, L.L.M. (1971) *Biochim. Biophys. Acta 239*, 113–119.
14. Taegtmeyer, H. (1985) *Circulation 72* (suppl. IV), 1–8.
15. Braunwald, E., and Sobel, B.E. (1988) in *Heart Disease: A Textbook of Cardiovascular Medicine* (Braunwald, E., ed.), pp. 1191–1221, W.B. Saunders Company, Philadelphia.
16. Krebs, H.A., and Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem. 210*, 33–66.
17. Langendorff, O. (1895) *Pflueger Arch. 61*, 291–332.
18. Robinson, J., and Newsholme, E.A. (1969) *Biochem. J. 122*, 449–453.
19. Hatch, G.M., and Choy, P.C. (1990) *Biochem. J. 268*, 47–54.
20. Holub, B.J., and Piekarski, J. (1976) *Lipids 11*, 251–257.
21. Rouser, G., Siakotos, A.N., and Fleisher, S. (1966) *Lipids 1*, 85–86.
22. Zhou, X., and Arthur, G. (1992) *J. Lipid Res. 33*, 1233–1236.
23. Carman, G.M., and Belunis, C.J. (1983) *Can. J. Microbiol. 29*, 1452–1457.
24. MacDonald, P.M., and McMurray, W.C. (1980) *Biochim. Biophys. Acta. 620*, 80–89.
25. Stahl, E. (1969) in *Thin-Layer Chromatography*, 2nd edn., pp. 48–49, Springer-Verlag Berlin, New York.
26. Schlame, M., and Hostetler, K.Y. (1992) *Methods Enzymol. 209*, 330–337.
27. Bradford, M.M. (1976) *Anal. Biochem. 72*, 248–254.
28. Whitehead, F.W., Trip, E., and Vance, D.E. (1981) *Can. J. Biochem. 59*, 38–47.
29. William, J.R., and Kobayashi, K. (1984) *Basic Res. Cardiol. 79*, 283–291.
30. Stryer, L. (1988) in *Biochemistry*, 3rd edn., pp. 605–609, W.H. Freeman and Company, New York.
31. Choy, P.C. (1982) *J. Biol. Chem. 257*, 10928–10933.
32. Paradies, G., and Ruggiero, F.M. (1988) *Biochim. Biophys. Acta 935*, 79–86.
33. Hostetler, K.Y. (1991) *Biochim. Biophys. Acta 1086*, 139–140.
34. Djavaheri-Mergny, M., Mora, L., Mazière, C., Auclair, M., Santus, R., Dubertret, L., and Maizière, J.C. (1994) *Biochem. J. 299*, 85–90.
35. Marks, D.I., Rockman, S.P., Oziemiski, M.A., and Fox, R.M. (1990) *J. Clin. Invest. 86*, 2080–2085.

[Received October 27, 1994, in revised form January 17, 1995; Revision accepted April 4, 1995]

Absorption of Triglycerides with Defined or Random Structure by Rats with Biliary and Pancreatic Diversion[1]

Michael Søberg Christensen[a,b,*], Anette Müllertz[c] and Carl-Erik Høy[a,b]

[a]Department of Biochemistry and Nutrition and [b]Center for Food Research, Technical University of Denmark, DK-2800 Lyngby, Denmark, and [c]Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark

ABSTRACT: Fat absorption may be compromised by pancreatic or bile insufficiency, resulting in low uptake of essential fatty acid and energy. Using a rat model of malabsorption, we examined the absorption of defined triglycerides with medium-chain fatty acids (MCFA) in the *sn*-1,3 positions and essential fatty acids in the *sn*-2 position (MLM) compared to other fats. The thoracic duct was cannulated for collection of lymph, and the common bile and pancreatic duct was cannulated to divert both the pancreatic juice and bile. The rats were given a single bolus of triglyceride as a taurocholate emulsion. Fat absorption was measured from collected lymph samples. The triglycerides administered were a defined triglyceride, MLM [mainly (8:0/10:0)-(18:2n-6)-(8:0/10:0)], a similar triglyceride subjected to chemical randomization, a mixture of medium-chain triglycerides and soybean oil, and soybean oil, respectively. The first three triglycerides had approximately 36 wt% linoleic acid (18:2n-6) content. Administration of defined triglyceride was followed by significantly higher lymphatic level (wt%) of 18:2n-6 ($P < 0.01$) as well as a relative enhancement in mol% of 18:2n-6 ($P < 0.05$) compared to the other triglycerides. Lymphatic absorption of MCFA was similar in the three first groups but not as efficient as for long-chain fatty acids. Our results indicate that defined triglycerides thus may provide a means to increase absorption of essential fatty acids in fat malabsorption, such as that seen in cystic fibrosis, or for pre-term infants.
Lipids 30, 521–526 (1995).

In the digestion of triglycerides (TG), lingual, gastric, and pancreatic lipases participate, of which under normal circumstances the pancreatic lipase is predominant. In certain patients, fat malabsorption may occur due to reduced or absent activity of pancreatic lipase, for instance, in cystic fibrosis in which the pancreatic duct may be occluded or in the pre-term infant with immature pancreas (1,2). In the treatment of cystic fibrosis, two strategies have been followed: (i) addition to the diet of major doses of protected hydrolytic enzymes (1); or (ii) addition to the diet of medium-chain triglycerides (MCT), which can be hydrolyzed very rapidly by lingual or gastric lipase, but do not contain essential fatty acids (EFA) (3). In order to include EFA in these TG, chemical interesterification of soybean oil and MCT has been used to produce TG which contain both MCFA and EFA, but with two-thirds of the EFA esterified in the *sn*-1,3 positions in contrast to normal fats (4,5). Since the lingual and gastric lipases are *sn*-3 specific and preferentially hydrolyze MCFA, these interesterified TG may not be optimal. By lipase-catalyzed *sn*-1,3 specific interesterification, it is, however, possible to produce TG in which EFA are maintained in the *sn*-2 position and MCFA are introduced into the *sn*-1,3 positions of the TG molecule.

In the present experiment, we used a rat model of pancreatic insufficiency and examined the absorption of various fats. We introduced a cannula into the thoracic lymph duct for collection of lymph containing the absorbed fat released as chylomicrons and a cannula into the common bile and pancreatic duct to divert bile and pancreatic juice from the intestine. We examined the absorption of four different fats: (i) A triglyceride with defined structure containing 18:2n-6 in the *sn*-2 position and MCFA in the *sn*-1,3 positions; (ii) a fat with similar fatty acid profile but random distribution of the fatty acids in the TG molecule as the presently used structured TG; (iii) A mixture of MCT and soybean oil; and (iv) soybean oil.

Our experiment demonstrates that in this model the best absorption of EFA is obtained from TG with defined structure.

MATERIALS AND METHODS

Animals. Male Wistar rats (Møllegaarden Breeding Laboratory, Ll. Skensved, Denmark) weighing 200–250 g, which had been raised on formula stock diet were fasted overnight (16 h) prior to surgery and then randomly divided into four groups.

Dietary fats. Defined TG with medium-chain, long-chain, medium-chain fatty acids (MLM), randomized MLM, a mixture of MCT and soybean oil, or soybean oil were used as bolus fat. The defined MLM and the randomized MLM were produced by Novo Nordisk A/S (Bagsvaerd, Denmark). The fatty acid composition and the structure of these oils (Table 1) were determined. Fatty acid profile was determined by

[1]Honored Student presentation, 84th AOCS Annual Meeting & Expo, Anaheim, California.

*To whom correspondence should be addressed at Department of Biochemistry and Nutrition, Building 224, Technical University of Denmark, DK-2800 Lyngby, Denmark.

Abbreviations: EFA, essential fatty acids; GLC, gas–liquid chromatography; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; MCT, medium-chain triglycerides; MLM, defined triglycerides with medium-chain, long-chain, medium-chain fatty acids; TG, triglyceride(s).

Copyright © 1995 by AOCS Press

TABLE 1
The Fatty Acid Compositions (wt%) and Structures of the Test Triglycerides[a]

Fatty acid	Structured MLM	Randomized MLM	MCT and soybean oil	Soybean oil
8:0	27.0	27.2	19.1	ND[a]
10:0	13.7	16.3	12.0	ND
16:0	2.2	2.0	7.3	10.6
18:0	0.8	0.7	2.5	3.6
18:1n-9	14.2	13.6	17.1	24.9
18:2n-6	37.8	35.6	36.3	52.7
18:3n-3	4.2	4.5	5.2	7.5
2-position				
8:0	ND	18.9	13.5	ND
10:0	ND	17.3	11.8	ND
16:0	2.5	2.6	0.4	0.6
18:0	ND	0.9	ND	ND
18:1n-9	24.9	14.6	16.8	23.4
18:2n-6	67.9	40.0	52.0	68.9
18:3n-3	6.6	4.7	5.4	7.1

[a]ND, not detected; MLM, defined triglycerides with medium-chain, long-chain, medium-chain fatty acids; MCT, medium-chain triglycerides.

gas–liquid chromatography (GLC). Fatty acid methyl esters were prepared with sodium methoxide (6). The fatty acid methyl esters were analyzed by GLC using a Hewlett-Packard 5890, series II (Waldbronn, Germany) chromatograph with a fused-silica capillary column (SP™-2380, 30 m × 0.32 mm i.d.; Supelco, Inc., Bellefonte, PA). Initial oven temperature was 70°C for 5 min followed by temperature programming at a rate of 15°C/min until 140°C, which was maintained for 2 min, and hereafter 3.5°C/min until 200°C. The final temperature was maintained for 18 min. Injection was on-column, and the carrier gas was helium at an initial flow rate of 2 mL/min. The fatty acid methyl esters were identified by GLC of authentic standards. TG structure was determined by regiospecific Grignard degradation followed by analysis of the 2-monoglycerides (7).

Fat emulsions were prepared by sonicating 0.5 mL fat with 0.5 mL of taurocholate (20 mM) plus 5 mg choline for 10 s (M.S.E. 150 watt Ultrasonic Disintegrator; M.S.E. Inc., Crawley, England. Settings: power: medium, Amplitude: 3). The emulsion was cooled with ice water during this procedure.

Surgical procedure. The experiments were approved by The Danish National Committee for Animal Experiments. Each animal was anesthetized with pentobarbital (8 mg/100 g body weight, i.p.), and after tracheostomy, the abdominal cavity was opened. A feeding tube was introduced orally about 2 cm into the stomach and secured with a ligature. The common bile and pancreatic duct was exposed close to the duodenum. A polyethylene catheter, 0.28 mm i.d. and 0.61 mm o.d., was inserted into the duct toward the liver and secured. The free end of the catheter was exteriorized. *Ductus thoracicus* was cannulated according to a modified procedure of Bollman *et al.* (8): The aorta was separated from the lymphatics cranially for the *cisterna chyli*. Approximately 0.8–1 cm of the duct was freed of connective tissue and two ligatures placed around the duct at a distance of 5 mm. The duct was partially transected and a U-shaped polyethylene catheter, 0.58 mm i.d. and 0.96 mm o.d., with a short bevel and prefilled with heparin (5000 i.U./mL), was inserted into the duct. The catheter was secured and exteriorized from the abdomen. The viscera was replaced *in situ* and the wound was closed. The rats were maintained at 25°C in anesthesia throughout the experiment.

Administration of oil emulsion and collection of lymph. After the surgery, the oil emulsion was administered intragastrically through the feeding tube followed by 1 mL of physiological saline, and the saline infusion was continued at a rate of 2 mL/h. The lymph was collected in tared tubes at 0°C containing 50 μL of a 10% (wt) solution of sodium EDTA. The collection of lymph commenced the moment the oil emulsion was administered and was pooled in 30-min fractions in the first 4 h and 60 min fractions in the following 4 h. The lymph samples were frozen immediately and kept at −20°C until further processing.

Lipid analysis. Fifty μL were taken from each fraction, and after addition of internal standard (methylpentadecanoate), the total lipid fraction was extracted according to Folch *et al.* (9). The solvent was evaporated under nitrogen, and the lipids resolved in 1 mL hexane. The fatty acid profile was determined as previously described for the dietary oils.

Data analysis. The data represent mean ± SEM. Two-way analysis of variance with the factors time and oil was used to test for significant differences between fatty acid wt% profiles. Student's *t*-test was used to test for significant variations in the total recovery and enhancement in mol% between groups. The absorption patterns were examined by fitting the best cubic polynomials to the means of the accumulated lymphatic transport.

RESULTS

The lymphatic absorption of various oils was examined in anesthetized rats subjected to cannulations of the thoracic duct and the common bile and pancreatic duct. Each sample

taken at a given time represented the total lymph collected between the previous collection and the designated time. The lymph flow was approximately 0.5 mL/h in all four groups.

The content of 18:2n-6 (wt%) in the lymph increased rapidly from the initial level of approximately 26% following administration of the oils and plateaued after 2 h (Fig. 1). The content was significantly higher ($P < 0.01$) after administration of defined MLM compared with the other oils. The plateau level of 18:2n-6 (wt%) in the lymph was 45.1 ± 1.6 (last sample) after administration of defined MLM, which was higher than the content of 18:2n-6 in the defined MLM administered (37.8%). After administration of randomized MLM or a mixture of MCT and soybean oil, the lymph contents plateaued at 40.7 ± 3.5 and 34.8 ± 2.4, respectively, which was approximately the same levels of 18:2n-6 as the contents of the administered oils. After administration of soybean oil, the content of 18:2n-6 increased from 26.7 ± 0.9 to 39.7 ± 2.2, which was much lower than in the soybean oil (53 wt% 18:2n-6).

As some MCFA were transported through the lymphatics, the wt% was converted to mol%, due to differences in molecular weight. The enhancement in mol% of 18:2n-6 (Fig. 2) was significantly greater following administration of defined MLM compared to the other sources ($P < 0.05$). For this calculation of the enhancement in mol%, the data for the 180–480-min period was pooled under the assumption that a steady state had been reached.

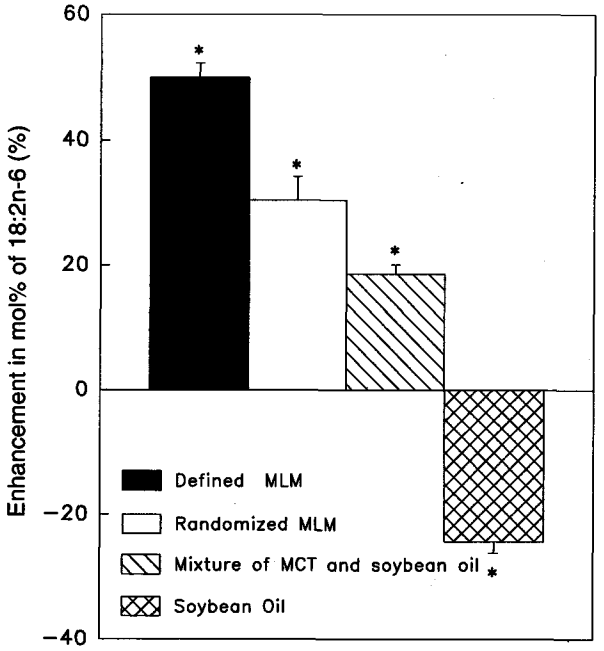


FIG. 2. The enhancement in mol% of 18:2n-6 in lymph total lipid fraction relative to the oil administered. The values represent mean ± SEM of the pooled data within each group from 180–480 min calculated as: [(mol% 18:2n-6 in the lymph/mol% 18:2n-6 in the oil) × 100 − 100]. Defined MLM, n = 39; randomized MLM, n = 42; mixture of MCT and soybean oil, n = 35; soybean oil, n = 28. *Significant differences at $P < 0.05$. See Figure 1 for abbreviations.

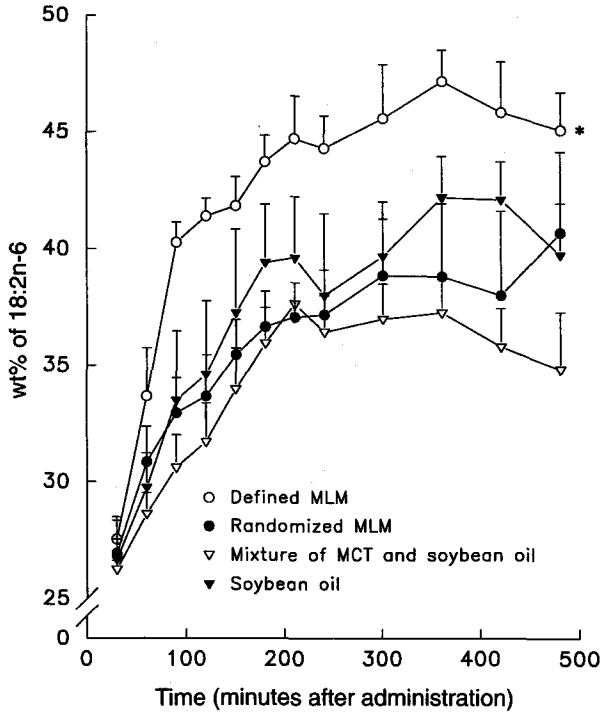


FIG. 1. Appearance of 18:2n-6 (wt%) in the lymph after administration of either defined triglycerides with medium-chain, long-chain, medium-chain fatty acids (MLM) (n = 6), randomized MLM (n = 6), mixture of medium-chain triglycerides (MCT) and soybean oil (n = 5), or soybean oil (n = 4). Values are expressed as mean ± SEM. *Significantly different from the three other groups at $P < 0.01$ by two-way analysis of variance.

The total recovery (percentages) of 18:2n-6 in the thoracic lymph was greater following administration of defined MLM compared to the three other types of TG which gave similar recoveries (Table 2).

TABLE 2
Recovery (% of administered amount of fatty acid)

	Fatty acid			
Administered fat	8:0	10:0	18:2n-6[a]	18:3n-3[b]
Structured MLM (n = 6)	0.8 ± 0.2	1.6 ± 0.4	9.9 ± 1.7	8.9 ± 1.9
Randomized MLM (n = 6)	0.7 ± 0.1	1.1 ± 0.2	5.7 ± 1.7	4.4 ± 1.0
MCT and soybean oil (n = 5)	1.3 ± 0.5	4.6 ± 1.6	6.6 ± 1.6	5.1 ± 1.6
Soybean oil (n = 4)	ND[c]	ND	3.7 ± 1.3	2.8 ± 1.3

[a]Significance level: for comparisons of 18:2n-6, $P = 0.07$ for structured MLM vs. randomized MLM; $P = 0.20$ for structured MLM vs. MCT and soybean oil; $P = 0.03$ for structured MLM vs. soybean oil.
[b]For comparisons of 18:3n-3, $P = 0.06$ for structured MLM vs. randomized MLM; $P = 0.17$ for structured MLM vs. MCT and soybean oil; $P = 0.05$ for structured MLM vs. soybean oil.
[c]ND, not detected. Data are expressed as mean ± SEM. *Student's t-test* was used to test for significant differences between the dietary groups. See Table 1 for abbreviations.

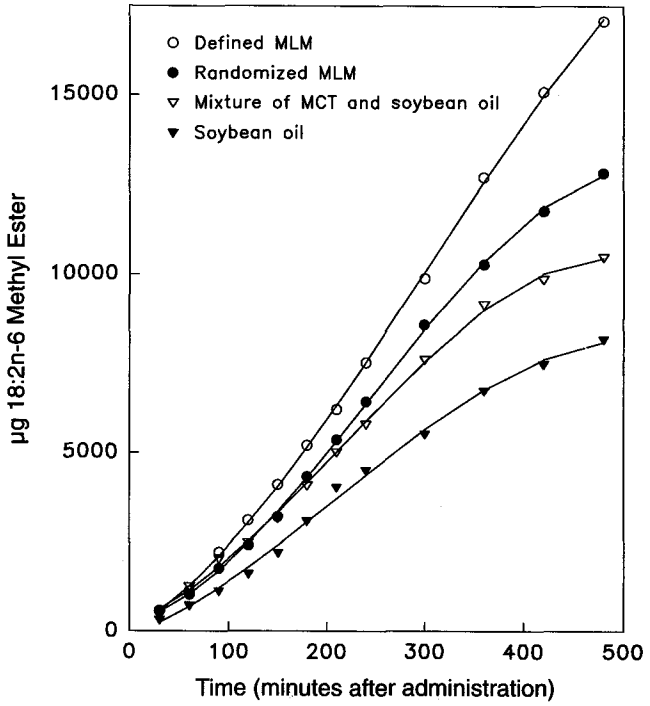


FIG. 3. The amount (μg) of 18:2n-6 transported through the lymphatics accumulated over the experimental period after administration of defined MLM (n = 6), randomized MLM (n = 6), mixture of MCT and soybean oil (n = 5), and soybean oil (n = 4). The curves indicate the best fitted cubic polynomials through the actual means (symbols). See Figure 1 for abbreviations.

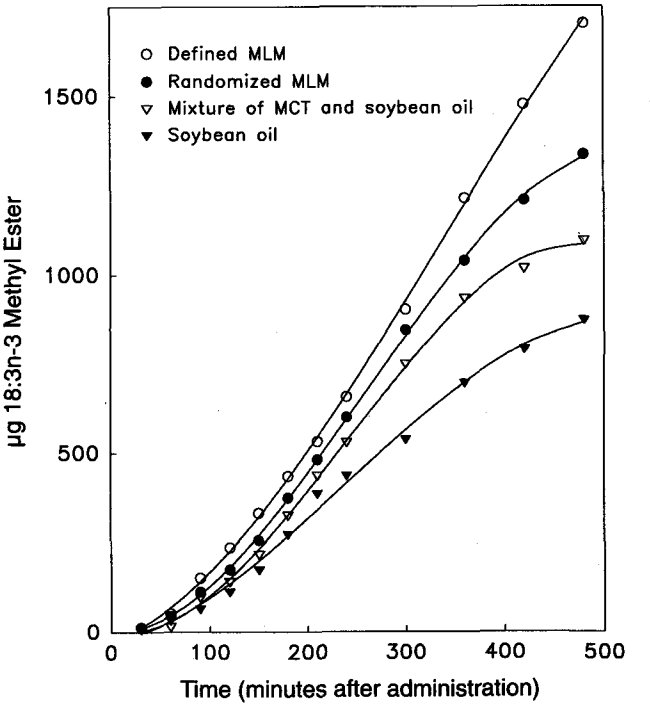


FIG. 4. The amount (μg) of 18:3n-3 transported through the lymphatics accumulated over the experimental period after administration of defined MLM (n = 6), randomized MLM (n = 6), mixture of MCT and soybean oil (n = 5), and soybean oil (n = 4). The curves indicate the best fitted cubic polynomials through the actual means (symbols). See Figure 1 for abbreviations.

The absorptions of 18:2n-6 and 18:3n-3 into the thoracic lymph as estimated from the amount (μg) accumulated in the lymph over the 8-h collection period are shown in Figures 3 and 4. The amount absorbed over the 8-h period was greater, and there seemed to be a progressive tendency beyond the 8-h for the increased absorption of both 18:2n-6 and 18:3n-3 from defined MLM compared to the other oils. This was evident from comparison of the slopes of the curves at the end of the experimental period: The absorption continued for defined MLM throughout the experimental period, whereas the slopes for the other three groups declined.

MCFA were also transported by the thoracic lymph duct but not as efficiently as long-chain fatty acid (LCFA) as seen by a relatively lower content of MCFA in the lymph lipids compared to the administered oils. The lymphatic transport of 10:0 was relatively higher compared to 8:0. The lymphatic levels of MCFA (maximal wt%) observed within the experimental period for 8:0 were 3.6 ± 0.8, 4.9 ± 0.9, 4.1 ± 1.3, not detected; and for 10:0, 7.8 ± 1.7, 9.0 ± 1.2, 6.9 ± 2.1, not detected, in defined MLM, random MLM, mixture of MCT and soybean oil, and soybean oil, respectively. There were no significant differences in the total lymphatic transport of MCFA between the three groups.

DISCUSSION

In this experiment, we examined the effect of TG structure and fatty acid profile on the intestinal absorption of linoleic acid. The study was performed in a rat model of pancreatic insufficiency, since obvious future applications of defined TG are in cases of malabsorption (10–12). The common bile and pancreatic duct was cannulated close to the entrance of the duodenum, and both the bile and the pancreatic juice were diverted. The absorption of fatty acids was monitored by collection of the total thoracic duct lymph which drains the chylomicrons secreted by the intestine.

In this model of pancreas insufficiency, the fatty acid absorption profiles demonstrated that EFA was better absorbed from defined TG compared to randomized TG, mixture of soybean oil and MCT, or soybean oil, although the last of these had a considerably higher content of 18:2n-6. The more efficient absorption of 18:2n-6 and 18:3n-3 from defined MLM is also evident from the accumulated 8-h absorptions. The absorption of EFA from defined MLM continued at a constant rate throughout the observed period, whereas the rate of absorption from the other fats declined toward the end of the experiment.

We administered the test emulsions intragastrically in order to reflect the hydrolysis due to the action of preduodenal lipases which are important in fat absorption during pancreatic insufficiency (1). In the rat, this lipase activity is mainly lingual, whereas gastric lipase is prominent in humans (2). In the present experiment, the esophageal secretion was obstructed, which previously was demonstrated to decrease intragastric hydrolysis of ingested fat by 90% (13). A normal

level of preduodenal lipases, irrespective of the origin, might result in a more significant difference in the absorption of defined MLM than observed in this experiment since these lipases favor degradation of TG with MCFA esterified in the *sn*-1,3 positions.

We did not monitor the portal vein transport of fatty acids. To determine the total absorption, this transport pathway should be taken into account, especially for MCFA but also for absorption of LCFA (14–16). It is well-established that the transport of MCFA by the portal vein is higher compared to LCFA in agreement with the present findings. However, the lymphatic transport of 10:0 was considerably greater compared to 8:0, especially when the content in the administered oil is taken into consideration. The major absorption pathway for 18:2n-6 is by the lymphatic system, irrespective of the experimental conditions considered (14), although the portal absorption becomes significant for low infusions (0.3–3 μmol/h) of 18:2n-6 (17). In the present experiment, the luminal load of 18:2n-6 was approximately a thousandfold higher and the average lymphatic output of 18:2n-6 was 60–70 μmol/h, demonstrating that the luminal load was adequate to make the lymphatic absorption the main pathway.

Endogenous fatty acids in the lymph predominantly originate from the bile (18) which was diverted in the present experiment. During lipid absorption, mucosal uptake of plasma free fatty acids and incorporation into intestinal TG is increased (19), but the dietary fatty acids are the major fatty acids of the lymph (20). The lymphatic transport of fat is reduced in bile-diverted rats as fat accumulates inside the enterocytes primarily due to the lack of biliary phosphatidylcholine (21,22). Previously, addition of choline has been demonstrated to have a similar influence on the fat transport, as adding egg yolk lecithin, and normalized the transport to the level of sham-operated rats (21). The fat absorption in our experiment was reduced fivefold compared to rats without common duct cannulation (23), indicating that a state of malabsorption had been established. Valdivieso and Schwabe (24,25) examined the influence of both pancreatic juice and bile on the absorption of trioctanoin and observed a 2.5–3-fold reduction after bile diversion and a 5–7-fold reduction after pancreatic atrophy in agreement with Bézard and Bugaut (26).

We observed lower lymphatic flow compared to experiments with unanesthetized rats (27), probably due to the influence of the anesthesia which in dogs has been demonstrated to reduce the lymph flow by 50% (28). Saline was infused at a rate of 2 mL/h, and bile and pancreatic juice were diverted at a rate of approximately 1 mL/h. The total absorption of fat was probably affected by the low lymph flow, but it should not influence the relative absorptions among parallel groups given different fats as the lymph flows were similar for all groups.

In a previous paper, Ikeda *et al.* (29) described the thoracic lymph absorption of 18:2n-6, 8:0, and 10:0 from various defined TG in rats with normal pancreatic lipase and bile secretion. They found higher absorption of 18:2n-6 from 8:0/18:2/8:0 than from 18:2/8:0/18:2 or from 18:2/18:2/18:2, and higher levels in the lymph of 8:0 and 10:0 when these fatty acids were located in the *sn*-2 position of the TG. However, they did not compare the absorption of fatty acids from TG with similar fatty acid profiles by defined or random structure. Our experiments demonstrate that when rats were fed TG with similar fatty acid profiles, the 18:2n-6 was better absorbed from a defined TG with 18:2n-6 in the *sn*-2 position. Furthermore, in our experiments we used defined TG and randomized TG, which were produced from soybean oil and MCT. Our experiment thus confirms the findings of Ikeda *et al.* (29) and extends them to include a state of reduced bile and pancreatic lipase levels and, furthermore, demonstrate the potential use of defined TG produced from commercially available fats.

We found that linoleic acid was considerably better absorbed from defined MLM than from soybean oil, in agreement with previous experiments by Jandacek *et al.* (30). They used rats with irrigated intestinal loop as a model for pancreatic insufficiency and demonstrated that 18:2n-6 was better absorbed from 8:0/18:2/8:0 than from 18:1/18:2/18:1. They did not, however, compare TG with similar fatty acid profiles and different structures. The relatively low content (wt%) of 18:2n-6 in the lymph observed after administration of soybean oil most likely reflects that soybean oil is absorbed less readily than oils containing MCFA either due to the emulsification properties or substrate specificities of the lipases in the gastrointestinal tract.

Jensen *et al.* (31) have demonstrated that TG with random structure based on MCT and fish oil are well absorbed into the lymph in dogs. They agreed with our results that the MCFA were well-absorbed from interesterified fats and from mixtures of MCT and polyunsaturated fat, but they detected no difference in absorption of polyunsaturated fatty acids. Recently, we have demonstrated in rats that defined TG with decanoic acid esterified in the *sn*-1,3 positions and n-3 polyunsaturated fatty acids in the *sn*-2 position were more readily absorbed compared to randomized TG with similar fatty acid composition (32) in agreement with the present findings in bile and pancreatic insufficient rats. Guillot *et al.* (33) also demonstrated lymphatic uptake of 8:0 and 10:0 in pancreatic-deficient pigs given a continuous duodenal infusion of MCT, indicating that these fatty acids are absorbed and that a considerable part will be released to the lymph.

Randomly esterified TG containing MCFA and 18:2n-6, which are frequently described in literature as structured lipids, are suitable sources of EFA and nonglucose energy for cystic fibrosis patients (5). Our study indicates that defined TG with MCFA in the *sn*-1,3 positions may be an even better fat source. This may result from the esterification of MCFA in the *sn*-1,3 positions since both the lipases in the gastrointestinal tract and preduodenal lipases are *sn*-1,3 specific and preferentially hydrolyze MCFA, resulting in rapid absorption of MCFA as free fatty acids and polyunsaturated fatty acids as 2-monoglyceride.

It has been hypothesized that MCT may be absorbed directly across the intestinal wall (34,35). The molecular

weights of structured MLM and the MCT used by Chow *et al.* (35) are similar to those in the present study, and, therefore, a contribution of structured TG taken up as such, or perhaps diglycerides, cannot be excluded.

Patients with cystic fibrosis may suffer from fat malabsorption caused by impaired secretion of pancreatic lipase (36). This may result in altered EFA status and in extreme cases may initiate EFA deficiency (37,38). Attempts to correct the EFA abnormalities using intravenous (39,40) or oral (41,42) administration of several types of lipid supplementation have been inconclusive.

In prolonged treatment, enteral administration of lipid supplementations must be preferred over intravenous administration. Defined MLM provides EFA as well as energy and should be considered as a possible dietary fat in the future treatment of malabsorption.

ACKNOWLEDGMENT

Marianne Jensen gave skillful technical assistance.

REFERENCES

1. Parsons, H.G. (1986) in *Fat Absorption* (Kuksis, A., ed.) Vol. I, pp. 261–293, CRC Press, Boca Raton.
2. Hamosh, M. (1990) *Lingual and Gastric Lipases: Their Role in Fat Digestion*, CRC Press, Boca Raton.
3. Bach, A.C., and Babayan, V.K. (1982) *Am. J. Clin. Nutr. 36*, 950–962.
4. Babayan, V.K. (1987) *Lipids 22*, 417–420.
5. Hubbard, V.S., and McKenna, M.C. (1987) *Lipids 22*, 424–428.
6. Christopherson, S.W., and Glass, R.L. (1969) *J. Dairy Sci. 52*, 1289–1290.
7. Becker, C.C., Rosenquist, A., and Hølmer, G. (1993) *Lipids 28*, 147–149.
8. Bollman, J.L., Cain, J.C., and Grindlay, J.H. (1948) *J. Lab. Clin. Med. 33*, 1349–1352.
9. Folch, J., Lees, M., and Sloane Stanley, G.H. (1956) *J. Biol. Chem. 226*, 497–509.
10. Heird, W.C., Grundy, S.M., and Hubbard, V.S. (1986) *Am. J. Clin. Nutr. 43*, 320–324.
11. Bell, S.J., Mascioli, E.A., Bistrian, B.R., Babayan, V.K., and Blackburn, G.L. (1991) *J. Am. Diet Assoc. 91*, 74–78.
12. Crosby, L.E., Swenson, E.S., Babayan, V.K., Blackburn, G.L., and Bistrian, B.R. (1990) *J. Nutr. Biochem. 1*, 41–47.
13. Hamosh, M., and Scow, R.O. (1973) *J. Clin. Invest. 52*, 88–95.
14. Bernard, A., Caselli, C., and Carlier, H. (1991) *Ann. Nutr. Metab. 35*, 98–110.
15. Hyun, S.A., Vahouny, G.V., and Treadwell, C.R. (1967) *Biochim. Biophys. Acta 137*, 296–305.
16. Bernard, A., and Carlier, H. (1991) *Experimental Physiology 76*, 445–455.
17. McDonald, G.B., Saunders, D.R., Weidman, M., and Fisher, L. (1980) *Am. J. Physiol. 239*, G141–G150.
18. Baxter, J.H. (1966) *J. Lipid Res. 7*, 158–166.
19. Gangl, A., and Ockner, R.K. (1975) *J. Clin. Invest. 55*, 803–813.
20. Savary, P., and Constatin, M.J. (1967) *Biochim. Biophys. Acta 144*, 549–555.
21. O'Doherty, P.J.A., Kakis, G., and Kuksis, A. (1973) *Lipids 8*, 249–255.
22. Tso, P., Balint, J.A., and Simmonds, W.J. (1977) *Gastroenterology 73*, 1362–1367.
23. Myrup Jensen, M., Christensen, M.S., and Høy, C.-E. (1994) *Ann. Nutr. Metab. 38*, 104–116.
24. Valdivieso, V.D., and Schwabe, A.D. (1965) *Gastroenterology 48*, 331–335.
25. Valdivieso, V.D., and Schwabe, A.D. (1965) *Gastroenterology 48*, 336–341.
26. Bézard, J., and Bugaut, M. (1986) in *Fat Absorption* (Kuksis, A., ed.) Vol. I, pp. 119–158, CRC Press, Boca Raton.
27. Chernenko, G.A., Barrowman, J.A., Kean, K.T., Herzberg, G.R., and Keough, K.M.W. (1989) *Biochim. Biophys. Acta 1004*, 95–102.
28. Schad, H., and Brechtelsbauer, H. (1977) *Pflügers Arch. 371*, 25–31.
29. Ikeda, I., Tomari, Y., Sugano, M., Watanabe, S., and Nagata, J. (1991) *Lipids 26*, 369–373.
30. Jandacek, R.J., Whiteside, J.A., Holcombe, B.N., Volpenhein, R.A., and Taulbee, J.D. (1987) *Am. J. Clin. Nutr. 45*, 940–945.
31. Jensen, G.L., McGarvey, N., Taraszewski, R., Wixson, S.K., Seidner, D.L., Pai, T., Yeh, Y.-Y., Lee, T.W., and DeMichele, S.J. (1994) *Am. J. Clin. Nutr. 60*, 518–524.
32. Christensen, M.S., Høy, C.-E., Becker, C.C., and Redgrave, T.G. (1995) *Am. J. Clin. Nutr. 61*, 56–61.
33. Guillot, E., Lemarchal, P., Dhorne, T., and Rerat, A. (1994) *Br. J. Nutr. 72*, 545–553.
34. Greenberger, N.J., Rodgers, J.B., and Isselbacher, K.J. (1966) *J. Clin. Invest. 45*, 217–227.
35. Chow, B.P.C., Shaffer, E.A., and Parsons, H.G. (1990) *Can. J. Physiol. Pharmacol. 68*, 519–523.
36. Shepherd, R.W., Cleghorn, G., Ward, L.C., Wall, C.R., and Holt, T.L. (1991) *Nutr. Res. Rev. 4*, 51–67.
37. Lloyd-Still, J.D., Johnson, S.B., and Holman, R.T. (1991) *Am. J. Clin. Nutr. 54*, 1029–1035.
38. Hubbard, V.S. (1983) *Eur. J. Pediatr. 141*, 68–70.
39. Elliott, R.B. (1976) *Pediatrics 57*, 474–479.
40. Chase, H.P., Cotton, E.K., and Elliott, R.B. (1979) *Pediatrics 64*, 207–213.
41. Rosenlund, M.L., Selekman, J.A., Kim, H.K., and Kritchevsky, D. (1977) *Pediatrics 59*, 428–432.
42. Chase, H.P., Welch, N.N., Rabaglia, M.E., and Dupont, J. (1982) *J. Pediat. Gastroenterol. Nutr. 1*, 49–54.

[Received September 25, 1993 and in final revised form March 29, 1995; Revision accepted April 7, 1995]

Sources of Triacylglycerol Accumulation in Livers of Rats Fed a Cholesterol-Supplemented Diet

Chuan-Hsiu Liu[a], Ming-ta Huang[b] and Po-Chao Huang[c,*]

[a]Department of Biochemistry, China Medical College, Taichung, [b]Department of Biochemistry, Chang Gung College of Medicine and Technology, Tao-yuan and [c]Department of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan

ABSTRACT: The source of free fatty acids (FFA) and the pathways contributing to the accumulation of neutral fats in livers of rats fed a cholesterol-enriched diet were investigated in this report. Supplementation with 1% cholesterol in the diet for four weeks resulted in hepatomegaly in the rats. The contents of cholesterol and triacylglycerols (TG) per gram liver measured in rats fasted overnight increased by 48 mg (~tenfold) and 66 mg (~fourfold), respectively. The activities of glycerophosphate acyltransferase and diacylglycerol acyltransferase, the two key enzymes for TG synthesis in liver microsomes, were found to increase by 23 and 19%, respectively, in the cholesterol-fed rats. The secretion of plasma TG present predominantly in very low density lipoprotein was found to decrease by ~30%. The incorporation of tritium from tritiated water in liver FFA increased by twofold in rats fed the cholesterol-supplemented diet, whereas the activity of CPT I in liver mitochondria decreased by 23%. The uptake of plasma FFA *in vivo* in livers of fasted rats maintained on the cholesterol-supplemented diet decreased by 60%. Our data thus indicate that the excess TG accumulated in livers of rats fed the cholesterol-enriched diet resulted from increased synthesis and decreased secretion of TG. To meet the demand of fatty acids for this purpose, *de novo* lipogenesis increased, whereas β-oxidation decreased. Although difference in the uptake of extrahepatic FFA may be discounted, a difference in the uptake of chylomicron remnants between the control and cholesterol-fed rats may not be ruled out.
Lipids 30, 527–531 (1995).

We have previously found that rats fed diets enriched in cholesterol often developed moderate to severe fatty livers (1,2). Cholesterol and triacylglycerols (TG) were found to accumulate in the liver (1,2). It has been suggested that the etiology of the fatty liver is related to essential fatty acid (EFA) deficiency (3), as linoleic acid was shown to be a preferred substrate for the esterification of cholesterol (4). EFA deficiency may impair the synthesis and function of phospholipids (PL) in the liver. Consequently, the ability to assemble and secrete very low density lipoprotein particles (VLDL) may be impaired. Recent reports by Fungwe *et al.* (5–7), however, showed that cholesterol-induced fatty liver was not necessarily related to EFA deficiency. Supplementing the cholesterol diets with a large quantity of corn oil (5–20%), a vegetable oil rich in linoleic acid, failed to prevent the accumulation of excess TG in the liver (6). Furthermore, the secretion of VLDL from the perfused liver was shown not to decrease, but rather to increase, in rats fed cholesterol-supplemented diets (7).

TG accumulation in the liver may be caused by increased synthesis of TG in the liver and/or decreased secretion of TG from the liver (5). The availability of free fatty acids (FFA) in the hepatocytes may be increased through an increase in *de novo* synthesis (7), an increase in uptake from extra-hepatic sources (6), and/or a decrease in β-oxidation (6). In this report, the roles of these metabolic pathways in contributing to cholesterol-induced fatty liver were characterized.

MATERIALS AND METHODS

Chemicals. L-[2-^{3}H]Glycerol 3-phosphate, disodium (10.6 Ci/mmol), [1-^{14}C]palmitoyl CoA (60 mCi/mmol), and tritiated water (1 Ci/mL) were purchased from Du Pont/New England Nuclear (Boston, MA). L-[methyl-^{3}H]Carnitine hydrochloride (74 Ci/mmol) was purchased from Amersham Corp (Arlington Heights, IL). Enzyme kits for the determination of cholesterol, TG, and FFA were purchased from Boehringer Mannheim (Mannheim, Germany). The kit for the determination of PL was purchased from bioMerieux Co. (Marcy-l'Etoile, France). Triton WR-1339 was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade.

Animals and diets. Male Wistar rats (190–210 g) were housed in a room with a 12 h light–dark cycle (light period: 7 a.m. to 7 p.m.). The animals were divided into two groups—one was fed a cholesterol-supplemented diet (1%) and the other the control diet for four weeks. The compositions of the control and cholesterol-supplemented diets are shown in Table 1. Lard and soybean oil were proportioned in the diets to yield a polyunsaturated/saturated fatty acid ratio of 1. De-

*To whom correspondence should be addressed at Department of Biochemistry, College of Medicine, National Taiwan University, 1 Jen-ai Rd, Sec. 1, Taipei, Taiwan.

Abbreviations: DGAT, diacylglycerol acyltransferase; EFA, essential fatty acids; FFA, free fatty acids; GAT, glycerophosphate acyltransferase; PL, phospholipids; TG, triacylglycerols; VLDL, very low density lipoproteins.

Copyright © 1995 by AOCS Press

TABLE 1
Composition of the Control and Cholesterol-Supplemented Diets

Ingredients	Control diet (%)	Cholesterol-supplemented diet (%)
Casein	20.0	20.0
Methyl cellulose	3.0	3.0
Vitamin mixture[a]	1.0	1.0
Salt mixture[a]	3.5	3.5
Choline chloride	0.095	0.095
Cholesterol	0	1.0
Lard	7.0	7.0
Soybean oil	3.0	3.0
DL-methionine	0.3	0.3
Sodium cholate	0.3	0.3
Corn starch	(making up to 100%)	

[a]AIN 76 vitamin and mineral mixtures.

pending on the protocol, the rats were either fed or fasted overnight at the time of the experiment. For rats fasted overnight, the food was removed at 5 p.m. and the experiment started at ~9 a.m. on the next day. The animals were allowed free access to drinking water at any time prior to the experiment.

Lipid analyses. Rats fasted overnight were sacrificed by decapitation. Blood was collected in tubes containing EDTA (1 mg/mL). Livers were excised and placed in ice-cold saline. The weight of the liver was determined after blotting dry. The livers were minced. Total lipids in the liver were isolated by the method of Folch *et al.* (8). The concentrations of cholesterol, PL, and TG were determined by the methods of Abell *et al.* (9), Stewart (10), and Soloni (11), respectively. Plasma was separated from blood cells by centrifugation. Plasma FFA were measured enzymatically (12).

Enzyme assays. Liver mitochondria were isolated from fed rats according to Harper and Saggerson (13). The microsomal fraction of liver homogenates was isolated according to Erickson *et al.* (14). The activity of mitochondrial CPT I was assayed by measuring the formation of [^{3}H] palmitoylcarnitine from [^{3}H] carnitine and palmitoyl CoA according to Saggerson *et al.* (15). The activity of microsomal diacylglycerol acyltransferase (DGAT) was measured by following the formation of [^{14}C] TG from [^{14}C] palmitoyl CoA and diacylglycerol according to Coleman and Bell (16). The microsomal activity of glycerol acyltransferase (GAT) was assayed by following the formation of [^{3}H]1-acylglycerol 3-phosphate from [^{3}H]glycerol 3-phosphate and palmitoyl CoA as described by Aarsaether *et al.* (17). Protein was determined by the method of Lowry *et al.* (18).

De novo synthesis of FFA. Fed rats were injected with 30 mCi [^{3}H]H_2O intraperitoneally one hour before sacrifice. *De novo* synthesis of FFA was measured according to Venkatesan *et al.* (19). Briefly, lipids were extracted from the liver and separated on thin-layer chromatography plates (Silica gel G) using a solvent system consisting of *n*-hexane, diethyl ether, and formic acid (80:20:2). Lipid fractions were visualized in an iodine chamber. FFA fractions were identified and scraped off the thin-layer chromatography plates. Radioactivity in FFA was determined in a Beckman (Palo Alto, CA) LS-250 β-counter.

TG secretion. A 20% solution of Triton WR-1339 was intravenously injected (50 mg/100 g body weight) to inhibit lipolysis of TG in the rats in the fasted state (20). The rats were sacrificed two hours after the injection as described by Swift *et al.* (21). Blood plasma was collected and VLDLs ($d < 1.006$ g/mL) were isolated by ultracentrifugation. Cholesterol, PL, and TG in VLDL, as well as in the unfractionated plasma, were measured by methods described previously.

Hepatic uptake of FFA. The uptake of plasma FFA in the liver *in vivo* was measured by an arteriovenous technique as described by Huang *et al.* (22). The animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg). Polyethylene cannulas (PE 10) were implanted in the aortas, portal veins, and hepatic vein (22). While the animals were being anesthetized, blood samples were taken sequentially from the hepatic veins, portal veins, and aortas through the implanted cannula into 1 c.c. tuberculin syringes previously rinsed with 5% EDTA. Hepatic uptake of FFA was determined by subtracting the concentration of FFA in the hepatic vein from that calculated for the hepatic influx (22). Hepatic influx concentration of FFA ($[FFA]_{inf}$) was calculated from the sum of the concentrations of FFA in the aorta ($[FFA]_a$), substituting for the hepatic artery, and the portal vein ($[FFA]_p$) multiplied by their respective fractional contributions of blood supply to the liver (i.e., $[FFA]_{inf} = 0.3 \times [FFA]_a + 0.7 \times [FFA]_p$). The fractional contributions of blood supply to the liver from the portal vein and the hepatic artery were previously determined to be 0.7 and 0.3, respectively (23). The purity of blood taken from the hepatic vein, free from vena caval contamination, was determined by the $p0_2$ method according to Huang (24).

Statistical analyses. Data were expressed as means ± SD. Statistical significance between data from rats fed the control and cholesterol-supplemented diets was determined by Student's *t*-test. A P value less than 0.05 was considered statistically significant.

RESULTS

Body and liver weight. Feed efficiency of the control and the cholesterol-supplemented diets was about the same. The gain in body weight was not significantly different between the two groups for a period of up to four weeks. Liver weight was, however, significantly increased in the group fed the high-cholesterol diet (12.4 ± 1.0 g vs. 8.4 ± 1.1 g in the control). Consequently, liver-to-body weight ratio increased in this group of rats.

Liver lipids. The content of cholesterol in the fasted state increased from ~5 mg/g liver in the control to ~53 mg/g liver in the cholesterol-fed rats (Table 2), and the content of TG increased from ~25 mg/g liver to ~90 mg/g liver (Table 2). The increases of cholesterol and TG in the liver were approximately ten- and fourfold, respectively, in the cholesterol-fed rats as compared to the controls. PL, on the other hand, de-

TABLE 2
The Contents of Cholesterol, Triacylglycerols, and Phospholipids in Livers of Rats That Had Been Fed Control or Cholesterol-Supplemented Diets for Four Weeks[a]

Diet	Cholesterol (mg/g liver)	Triacylglycerols (mg/g liver)	Phospholipids (mg/g liver)
Control	4.8 ± 0.5	24.5 ± 6.2	31.7 ± 2.3
Cholesterol	53.1 ± 7.8[b]	90.3 ± 16.9[b]	26.9 ± 2.3[b]

[a]Data are means ± SD of six experiments for both groups, which were fasted overnight prior to sacrifice.
[b]$P < 0.05$, vs. control.

creased (13%) on a per gram liver basis in the cholesterol-fed rats, as compared to the controls (Table 2). On the whole liver basis, cholesterol feeding increased the content of cholesterol in the liver by 14.6-fold, TGs by 5.1-fold, and PLs by 1.3-fold.

TG synthesis. The activities of the two key enzymes involved in TG synthesis, GAT and DGAT, were measured in liver microsomes isolated from rats fed the control and the cholesterol-supplemented diets in the fed state. The activity of GAT, which catalyzes the first esterification step of TG synthesis, increased 23% in the cholesterol-fed rats (Table 3). The activity of DGAT, which catalyzes the conversion of diacylglycerols to TG, increased by 19% in the cholesterol-fed rats, as compared to the controls (Table 3).

β-Oxidation of FFA. The activity of CPT I in isolated mitochondria, the rate-limiting enzyme for β-oxidation, was found to decrease significantly by 23% in the fed state in rats fed the cholesterol-supplemented diet, as compared to the controls (Table 3).

De novo *synthesis of FFA.* The incorporation of tritiated water injected intraperitoneally in fatty acids in the liver was 6,544 ± 1,480 dpm/h/g in the control and 13,240 ± 2,663 dpm/h/g in the cholesterol-fed rats. The rate of *de novo* synthesis of FFA thus increased by twofold in the cholesterol-fed rats as compared to the controls.

Hepatic secretion of lipids. Total and VLDL lipid secretion were measured in the fasted state. Triton WR-1339 was injected intravenously to block the activity of lipoprotein lipase. The contents of lipids in the unfractionated plasma and in VLDL before and after two hours of injection are shown, respectively, in Tables 4 and 5. No significant difference in

TABLE 3
Activities of Microsomal Glycerophosphate Acyltransferase (GAT), Diacylglycerol Acyltransferase (DGAT), and CPT-I in Livers of Rats Fed Control or Cholesterol-Supplemented Diet for Four Weeks[a]

Diet	GAT[b]	DGAT[c]	CPT-I[d]
Control	1.13 ± 0.16	3.36 ± 0.36	9.67 ± 1.06
Cholesterol	1.39 ± 0.08[e]	4.01±0.58[e]	7.46 ± 0.84[e]

[a]Data are means ± SD. Numbers of experiments in each groups are 8.
[b]Expressed as nmol of 1-acylglycerol 3-phosphate formed/min/mg microsomal protein.
[c]Expressed as nmol of triacylglycerol formed/min /mg microsomal protein.
[d]Expressed as nmol of palmitoyl–CoA formed/min/mg mitochondrial protein.
[e]$P < 0.05$, vs. control.

TABLE 4
Total Triacylglycerols, Cholesterol, and Phospholipids in Plasma Before and After Injection of Triton WR-1339 in Rats That Had Been Fed Control or Cholesterol-Supplemented Diet for Four Weeks[a]

Diet	Control	Cholesterol-fed
Triacylglycerols		
Before	104.7 ± 39.0	80.5 ±17.4
After	785.1 ± 86.9	574.8 ± 88.8[b]
Cholesterol		
Before	82.5 ± 10.5	85.5 ± 10.5
After	156.6 ± 17.2	345.2 ± 24.2[b]
Phospholipids		
Before	126.5 ± 13.0	105.6 ± 6.2[b]
After	91.7 ± 12.9	182.5 ± 23.4[b]

[a]Data are means ± SD. Units are mg/dL. Rats from each dietary group were divided into two groups for the experiments—after fasting overnight, one was sacrificed before, and the other two hours after intravenous injection of Triton WR-1339 (50 mg/100 g body weight). Numbers of experiments for both the control and cholesterol-fed rats were six before the injection, and eight after the injection.
[b]$P < 0.05$, vs. control.

TG concentration was found between VLDL and the unfractionated plasma before the injection of Triton in the two groups. After the injection, plasma TG, found to be associated predominantly with VLDL, increased approximately sevenfold in both groups of rats. The rate of increase in plasma TG concentration in the cholesterol-fed rats was, however, ~40% less than that in the controls. VLDL cholesterol was 13% of the total in the plasma in the control and 29% in the cholesterol-fed rats before the injection of Triton. The proportion of plasma cholesterol in VLDL increased to 60% in the cholesterol-fed and 72% in the control rats after the injection of Triton. The increases in the concentration of cholesterol were 1.9–4.0-fold in the plasma and 8.2–11-fold in VLDL after the injection of Triton. VLDL–PL as a percentage of total plasma PL was ~15% in the two groups before

TABLE 5
VLDL-Triacylglycerols, Cholesterol, and Phospholipids Before and After Injection of Triton WR-1339 in Rats That Had Been Fed Control or Cholesterol-Supplemented Diet for Four Weeks[a]

Diet	Control	Cholesterol-fed
Triacylglycerols		
Before	78.0 ± 32.1	58.0 ±14.0
After	774.5 ± 86.9	550.0 ± 85.3[b]
Cholesterol		
Before	10.9 ± 4.1	25.2 ± 9.4[b]
After	112.2 ±18.5	207.5 ± 3.3[b]
Phospholipids		
Before	16.4 ± 6.4	18.2 ± 5.5
After	112.4 ± 9.5	139.6 ± 11.1[b]

[a]Data are means ± SD. Units are mg/dL. Rats from each dietary group were divided into two groups for the experiments—after fasting overnight, one was sacrificed before, and the other two hours after intravenous injection of Triton WR-1339 (50 mg/100 g body weight). Numbers of experiments for both the control and cholesterol-fed rats were six before the injection, and eight after the injection.
[b]$P < 0.05$, vs. control.

the injection. After the injection, the concentration was not changed in the control, but was increased 73% in the cholesterol-fed rats. VLDL–PL as a percentage of the total in the plasma was increased to 75% in the cholesterol-fed rats and 100% in the control rats after the injection of Triton.

Hepatic gradients of FFA. The uptake of extra-hepatic FFA in the liver was measured by arteriovenous difference technique in intact rats (22). In the fasted state, the concentration of FFA in the aorta was 0.74 ± 0.17 mM (n = 5) in rats fed the cholesterol-supplemented diet and 0.96 ± 0.19 mM (n = 4) in rats fed the control diet. The difference between the two was not statistically significant. The arteriovenous gradient of FFA across the liver in the control rats was, however, 100% greater than that found in the cholesterol-fed rats (0.78 ± 0.37 vs. 0.39 ± 0.18 mM). This indicates that the uptake of plasma FFA in the liver was 50% less in rats fed the cholesterol-supplemented diet than the controls in the fasted state, assuming that hepatic blood flow was not significantly different between the two groups.

DISCUSSION

In this study, we showed that supplementation of 1% cholesterol in the diet for four weeks stimulated the synthesis and accumulation of TG in the liver. Because the polyunsaturated/saturated fatty acid ratio of the dietary fat was set to be 1, following the dietary guideline of the National Science Council (Washington, D.C.), the supply of the polyunsaturated fatty acids to the rats was deemed sufficient. The incidence of the fatty liver was thus not a result of a deficiency in EFA (3,4) or a result of impaired PL synthesis (3,4), as was suggested by the diminished PL content in rats fed the cholesterol-supplemented diet on the basis of per gram tissue weight (Table 2). On the basis of the whole liver, PL content in the cholesterol-fed rats was found to increase by 30%. Furthermore, the secretion of PL in VLDL was found to increase in rats fed the cholesterol-supplemented diet.

The amounts of TG and cholesterol accumulated in the liver in the current study were three- to fivefold greater than those reported by Fungwe *et al.* (6). These discrepancies may be attributed to differences in the dietary regimen in the two studies and the duration during which the animals were maintained on the diets. Fat content in our diet was 10% rather than 5% as in the study of Fungwe *et al.* (6). Cholesterol contents in the diets in our study and that of Fungwe *et al.* (6) were 1 and 0.5%, respectively. The animals in our study were fed the diet for four weeks, while those of Fungwe *et al.* (6) were fed for one week. According to Fungwe *et al.* (6), changing dietary fat content alone from 5 to 15% can result in a threefold increase in liver TG and a twofold increase in liver cholesteryl esters.

While Fungwe *et al.* (5) showed that the secretion of VLDL-TG increased in the isolated perfused liver, we found that *in vivo* the secretion of TG by the liver determined in the plasma or VLDL decreased in rats fed the cholesterol-supplemented diet, as compared to the controls. Plasma TG was found predominantly in VLDL both before and after the injection of the inhibitor for lipoprotein lipase, Triton WR 1339, in either the control or cholesterol-fed rats (Tables 4 and 5). Very little TG was found to be associated with lipoproteins other than VLDL.

In contrast, the concentration of cholesterol increased 3.5-fold in the plasma and 1.8-fold in VLDL in the cholesterol-fed rats, as compared to the controls (Tables 4 and 5). The majority of the cholesterol in the plasma was found to be present in lipoproteins other than VLDL before the injection of Triton WR 1339. After the injection, the percentage of cholesterol in VLDL increased. A significant proportion of plasma cholesterol remained in lipoprotein fractions other than VLDL. The rate of accumulation of cholesterol in non-VLDL lipoproteins was greater in the cholesterol-fed rats than in the controls, suggesting that the turnover of cholesterol and cholesteryl esters in non-VLDL lipoproteins was increased in the cholesterol-fed rats.

Like cholesterol, the secretion of PL also increased in the cholesterol-fed rats (Tables 4 and 5). Blockade of the activity of lipoprotein lipase resulted in no additional PL secretion in the control, but about 73% more PL in the cholesterol-fed rats. The percentage of plasma PL in VLDL increased in both groups of rats following the injection of Triton. Before the injection of Triton WR 1339, only a small fraction of plasma PL was found to be associated with VLDL. After the injection, almost all of the PL in the control and 75% in the cholesterol-fed rats was found in VLDL. Thus, it seems that inhibition of lipoprotein lipase could result in the redistribution of PL in the lipoproteins.

No attempt was made to differentiate the unesterified and esterified forms of cholesterol in this study. Given the magnitude of the increase in total cholesterol in VLDL (85%), both forms of cholesterol can be expected to increase. Fungwe *et al.* (5) showed that the increase in plasma cholesterol can be found mainly in the esterified rather than the unesterified fraction of cholesterol. Because the increases in the amounts of surface lipids (PL and cholesterol) in the cholesterol-fed rats were small (~10%), VLDL from this group of rats and that from the control rats were not expected to be different in size. Both TG and cholesteryl esters are located in the core of VLDL particles. Constrained by particle size, excess secretion of cholesteryl esters may result in less space for TG to be incorporated in VLDL (25). This has been demonstrated in *in vitro* experiment. When isolated hepatocytes were incubated in a medium enriched in cholesterol, the secretion of cholesteryl esters increased and TG decreased in VLDL (26).

The other question to be addressed in this report is the source of FFA for TG synthesis in the liver. We found that *de novo* synthesis of FFA increased, and the degradation of FFA in the liver decreased in rats fed the cholesterol-supplemented diet, in agreement with the findings of Fungwe *et al.* (5–7). In contrast to the experiment of Fungwe *et al.* (6), hepatic uptake of FFA was measured *in vivo* instead of *in vitro* in the isolated perfused liver (6). Whereas the uptake of plasma FFA in the isolated perfused liver was not affected by

dietary cholesterol (6), the uptake *in vivo* as indicated by the arteriovenous gradient of FFA across the liver decreased by 60%. Both studies nevertheless ruled out plasma FFA as a source for excess TG synthesis in the liver. It appears that the *de novo* synthesized FFA may be sufficient to account for the TG accumulated in the liver in the cholesterol-fed rats. As the supply of cholesterol is abundant, it is not necessary to maintain 3-hydroxy-3-methylglutaryl–CoA reductase in an active state. More acetyl CoA may be available for lipogenesis (7). To prevent futile cycling between lipogenesis and FFA oxidation, the activity of mitochondrial CPT I diminished (6).

Another source of extrahepatic FFA for TG accumulation in cholesterol-fed rats is cholesteryl esters contained in the hydrophobic cores of apolipoprotein C-depleted VLDL and chylomicron remnants (27). Cholesteryl esters in these lipoprotein particles can be transferred preferentially to the liver through interactions at the cell surface between these particles, hepatic lipase, and low-density lipoprotein receptor before the irreversible uptake of the lipoprotein particles themselves (27,28). The half-life of cholesteryl esters in the liver is short (~15 min) (27). Fatty acids released through the hydrolysis of cholesteryl esters in the liver were found to be incorporated in glycerolipids (27). The exact role for this pathway in TG accumulation in the cholesterol-fed rats was not determined in this report.

The increases in the activities of GAT and DGAT in livers of the cholesterol-fed rats may be secondary to the increase in fatty acid synthesis. The increases in DGAT activity and TG synthesis in primary culture of hepatocytes were found to be associated with an increase in the availability of FFA in the medium (29). Our data from the arteriovenous studies, however, indicate that newly synthesized FFA is more effective than plasma FFA in serving as the substrate for TG synthesis in the liver. The effectiveness of endogenous FFA in promoting TG synthesis has also been shown by Fungwe *et al.* (6). These investigators found that although the incorporation of [^{14}C] oleate in TG increased in the isolated perfused liver, the uptake of [1-^{14}C] oleate was not significantly altered in the cholesterol-fed rats.

In conclusion, our results support the roles of increased lipogenesis, decreased oxidation of fatty acids and decreased secretion of VLDL as causes for the accumulation of TG in the liver in the cholesterol-fed rats. Of the two extra-hepatic sources of fatty acids, albumin-bound FFA may be discounted for the difference in TG accumulation in the livers between the control and cholesterol-fed rats. A difference in the uptake of esterified fatty acids in chylomicron remnants, however, remains a possibility.

ACKNOWLEDGMENTS

We would like to thank C.C. Chao for technical assistance. This work was supported by grants (NSC-80-0412-B002-42, NSC-81-0412-B002-112, and NSC-82-0412-B182-26) from the National Science Council of the ROC government in Taiwan.

REFERENCES

1. Chen, L.C., Hsu, H.S., and Huang, P.C. (1980) *J. Chinese Nutr. Soc. 5,* 55–66.
2. Lin, D.S., and Huang, P.C. (1984) *J. Chinese Nutr. Soc. 9,* 47–55.
3. Mayes, P.A. (1993) in *Harper's Biochemistry,* 23rd edn. (Murray, R.K., Granner, D.K., Mayes, P.A., and Rodwell, V.W., eds.), pp. 259, Appleton and Lange.
4. Ueno, K., and Okuyama, H. (1986) *Lipids 21,* 475–480.
5. Fungwe, T.V., Cagen, L., Wilcox, H.G., and Heimberg, M. (1992) *J. Lipid Res. 33,* 179–191.
6. Fungwe, T.V., Cagen, L.M., Cook, G.A., Wilcox, H.G., and Heimberg, M. (1993) *J. Lipid Res. 34,* 934–941.
7. Fungwe, T.V., Fox, J.F., Cagen, L.M., Wilcox, H.G., and Heimberg, M. (1994) *J. Lipid Res. 35,* 311–318.
8. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497–509.
9. Abell, L.L., Levy, B.B., Brodie, B.B., and Kendall, F.E. (1952) *J. Biol. Chem. 195,* 357–367.
10. Stewart, J.C.M. (1980) *Anal. Biochem. 104,* 10–14.
11. Soloni, F.G. (1971) *Clin. Chem. 17,* 529–534.
12. Shimizu, S., Tani, Y., Yamada, H., Tabata, M., and Murachi, T. (1980) *Anal. Biochem. 107,* 193–198.
13. Harper, R.D., and Saggerson, E.D. (1975) *Biochem. J. 152,* 485–494.
14. Erickson, S.K., Shrewsbury, M.A., Brooks, C., and Meyer, D.J. (1980) *J. Lipid Res. 21,* 930–941.
15. Saggerson, E.D., Carpenter, C.A., and Tselentis, B.S. (1982) *Biochem. J. 208,* 667–672.
16. Coleman, R., and Bell, R.M. (1976) *J. Biol. Chem. 251,* 4531–4543.
17. Aarsaether, N., Berge, R.K., Aarsland, A., Svardal, A., and Ueland, P.M. (1988) *Biochim. Biophys. Acta. 958,* 70–80.
18. Lowry, O.H., Rosebrough, N.J., Forr, A.C., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
19. Venkatesan, S., Ward, R.J., and Peters, T.J. (1987) *Clinical Science 73,* 159–163.
20. Otway, S., and Robinson, D.S. (1967) *J. Physiol. 190,* 321–332.
21. Swift, L.L., Soul'e, P.D., and LeQuire, V.S. (1982) *J. Lipid Res. 23,* 962–971.
22. Huang, M.-T., Chen, M.-Y., Liu, H.-Y., and Shih, H.-P. (1994) *Life Sci. 55,* 999–1007.
23. Richardson, P.D.I., and Withrington, P.G. (1981) *Gastroenterology 81,* 159–173.
24. Huang, M.-T. (1991) *J. Appl. Physiol. 71,* 359–364.
25. Gibbons, G.F. (1990) *Biochem. J. 268,* 1–13.
26. Davis, R.A., Malone-McNeal, M., and Moses, R.L. (1982) *J. Biol. Chem. 257,* 2634–2640.
27. Zammit, V.A., and Moir, A.M.B. (1994) *Trends Biochem. Sci. 19,* 313–317.
28. Holder, J., Zammit, V.A., and Robinson, D.S. (1990) *Biochem. J. 272,* 735–741.
29. Haagsman, H.P., and Van Golde, L.M.G. (1981) *Arch. Biochem. Biophys. 208,* 395–402.

[Received September 14, 1994, and in revised form March 13, 1995; Revision accepted April 28, 1995]

Effects of Highly Hydrogenated Soybean Oil and Cholesterol on Plasma, Liver Cholesterol, and Fecal Steroids in Rats

Masaharu Kamei[a,*], Sumiko Ohgaki[a], Tamotsu Kanbe[b], Isao Niiya[c], Haruo Mizutani[d], Isao Matsui-Yuasa[e], Shuzo Otani[f] and Shigeru Morita[a]

[a]Laboratory of Nutritional Physiology, Osaka City Institute of Public Health and Environmental Sciences, Osaka 543, Japan, [b]Department of Domestic Sciences, Faculty of Education, Miyazaki University, Miyazaki 889-21, Japan, [c]Japan Institute of Oil and Fats, Other Foods Inspection, Foundation, Tokyo 103, Japan , [d]Ueda Oils and Fats Manufacturing Co., Kobe 658, Japan, [e]Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, Osaka 558, Japan and [f]Department of Biochemistry, Osaka City University, Medical School, Osaka 545, Japan

ABSTRACT: We investigated the relationship between dietary highly hydrogenated soybean oil (HSO) and cholesterol transport in rats. In the first study, to examine the effects on cholesterol transport of different concentrations of HSO in dietary oil, rats were given one of the three diets containing 0, 25, or 50% HSO in dietary oil with cholesterol (5 g/kg diet) or a diet without HSO and cholesterol for 22 d. Feeding the high concentration of HSO prevented the increase in plasma total cholesterol, hepatic total lipids, and cholesterol and the decrease in high-density lipoprotein-cholesterol, which were caused by dietary cholesterol. Moreover, HSO increased the fecal excretion, fecal lipids, and steroids in a dose-dependent manner. In the second study, to examine the effects on cholesterol transport of redistribution of stearic acid in the triacylglycerol species contained in HSO, rats were given one of the six diets containing HSO (distearoylmonoacylglycerol and tristearoylglycerol)-rich, monostearoylglycerol-rich, or palmitic acid-rich oil with/without cholesterol (5 g/kg diet), for 30 d. Whereas the accumulation of cholesterol in the body was reduced, cholesterol excretion was enhanced effectively in rats given the HSO-rich diet compared with rats given the monostearoylglycerol-rich diet. These results suggested that not only the high concentration of stearic acid but also its uneven distribution in HSO–triacylglycerol contributed to the reduction in intestinal cholesterol absorption in rats.

Lipids 30, 533–539 (1995).

Epidemiological studies have shown that plasma cholesterol affects the development of arteriosclerosis. Keys *et al.* (1) and Hegsted *et al.* (2) have shown that the fatty acid composition in dietary fat affected the concentration of plasma cholesterol. Their reports confirmed that the effect of saturated fatty acids on plasma cholesterol is altered by their chain length. To reduce the risk of coronary heart disease, several official recommendations have suggested a reduction of saturated fatty acid and cholesterol intake. However, among the saturated fatty acids, stearic acid is not hypercholesterolemic (3–6). Michel *et al.* (7) and Apgar *et al.* (8) have reported the poor absorption of this fatty acid from cocoa butter. Grigor and Dunkley (9) have also reported the low absorptivity of stearic acid from an investigation of the origin of this fatty acid in feces. Considering these reports and others (10,11), dietary stearic acid might prevent cholesterol absorption by promoting cholesterol excretion as dietary fiber does. However, there are some conflicting reports about stearic acid absorption (4,12), which have demonstrated that it is not so difficult to absorb, despite its high melting point. Therefore, the controversy has remained regarding the digestibility of stearic acid, which might be affected by its distribution in triacylglycerols.

During post-absorption, Bonanome and Grundy (4) have shown that stearic acid is rapidly desaturated to oleic acid, which may have indicated a neutral or slightly hypocholesterolemic effect. They suggested that this reaction might be a regulatory mechanism of the stearic acid effect on plasma cholesterol level.

In highly hydrogenated vegetable oils, most of C-18 unsaturated fatty acids have been converted to stearic acid. A large amount of stearic acid unevenly distributed in hydrogenated oils might have different nutritional effects compared with a similar amount of this fatty acid distributed uniformly in natural fats or interesterified hydrogenated oils. One of these effects, for example, might be interference with absorption of other dietary components. Therefore, we investigated whether highly hydrogenated soybean oil (HSO) influences the absorption and transport of dietary cholesterol in rats.

MATERIALS AND METHODS

Animal experiments. Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were used throughout the experiments. Animals weighing about 80 g were housed in cages with a

*To whom correspondence should be addressed at Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543, Japan.

Abbreviations: Chl, Cholesterol (in diets); HSO, highly hydrogenated soybean oil; HDL, high-density lipoprotein.

Copyright © 1995 by AOCS Press

wire-screen floor and were fed with a commercial, nonpurified diet (Clea Japan, Inc., Tokyo, Japan) for 5 d before the experiment. Room temperature was maintained at 22–24°C with a 12-h cycle of light (1300–0100) and dark (0100–1300). In Experiment 1, pre-feeding rats were divided into four groups of six animals each and were given the experimental diets for 22 d. In Experiment 2, after pre-feeding, rats were divided into six groups of six animals each and were given the experimental diets for 30 d. Animals were provided food and water *ad libitum* in both experiments. Feces collected for 48 h just before fasting were lyophilized and frozen at −70°C until assay. At the end of the experimental period, after fasting overnight, the rats were anesthetized with diethyl ether and sacrificed between 0700–0800. Blood was collected from the abdominal aorta, using a plastic syringe containing heparin, for plasma cholesterol analysis. Immediately after the livers were removed, weighed, and cooled on ice, they were frozen at −70°C until assay. All aspects of the animal experiment were approved by the Animal Policy and Welfare Committee in our Institute.

Diets in Experiment 1. The composition of the basal diet and the fatty acids in the dietary fats are shown in Tables 1 and 2. Soybean oil was the control dietary fat. Experimental dietary fats were prepared by replacing 25 or 50% of the soybean oil with HSO, which was a gift from Ueda Oils & Fats Manufacturing Co. (Kobe, Japan). These fats were named HSO25 and HSO50, respectively. To prepare diets containing cholesterol, i.e., the control diet, the cholesterol-containing control diet, the HSO25-Chl diet (where Chl = cholesterol), and the HSO50-Chl diet, cholesterol and sodium–cholate were added to each diet to final concentrations of 0.5 and 0.05%, respectively.

TABLE 1
Composition of Experimental Diets

Diet (g/kg diet)	Cholesterol-containing	Cholesterol-free
Milk casein	200	200
α-Corn starch	240.5	245.5
Sucrose	200	200
Fat[a]	200	200
Cellulose powder	100	100
AIN-Mineral mix	40	40
AIN-Vitamin mix	9	9
Methionine	3	3
Choline-Cl	2	2
Cholesterol	5	0
Na-Cholate	0.5	0.5

[a]In Experiment 1, fats applied to each experimental diet were as follows: soybean oil in the control diet, highly hydrogenated soybean oil (HSO)/soybean oil (1:3) in the HSO25-Chl diet and HSO/soybean oil (1:1) in the HSO50-Chl diet. The fatty acid composition of each fat is shown in Table 2. The HSO25-Chl diet and HSO50-Chl diet were supplemented with cholesterol as well as the cholesterol-containing control diet. In Experiment 2, applied fats were prepared by mixing in some oils to modulate the fatty acid composition comparably. The proportion of oils and the fatty acid composition of each fat is shown in Table 3. Milk casein, α-corn starch, cellulose powder, mineral mix, and vitamin mix were purchased from the Oriental Yeast Co. (Tokyo, Japan); Chl, cholesterol.

TABLE 2
Fatty Acid Composition in Dietary Fats/Oil Applied on Experiment 1[a]

	Control (%)	HSO25-Chl (%)	HSO50-Chl (%)
16:0	10.6	8.5	6.8
18:0	3.8	25.0	46.8
18:1	23.9	18.5	13.1
18:2	53.2	41.0	28.0
18:3	7.5	5.6	4.0
20:0	0.5	0.7	0.7
22:0	0.6	0.7	0.6
Total saturated	15.5	34.9	54.9

[a]Soybean oil was used as the control oil. HSO25 and HSO50 were prepared by mixing soybean oil and HSO at the ratio of 3:1 and 1:1, respectively. HSO was a gift from Ueda Oil Co.(Kobe, Japan). Soybean oil was obtained from a local market. The fatty acid composition was determined by gas–liquid chromatography as detailed in the text. The melting point of HSO was 65.7°C and the iodine value was 1. See Table 1 for abbreviations.

Diets in Experiment 2. The composition of the experimental basal diet was the same as that discussed for Experiment 1 except for fat composition. As shown in Table 3, the fatty acid composition of three dietary fats was adjusted to nearly the same amount. The triacylglycerol profile of HSO analyzed by the procedure described by Litchfield (13) consisted of tristearoylglycerol 57% and distearoylmonoacylglycerol 43%. HSO-rich fat (Fat-B) was prepared by mixing HSO, olive oil, and safflower oil at the ratio of 4.5:5:0.5, respectively. Monostearoylglycerol-rich fat (Fat-C) was prepared by mixing monostearoylglycerol (the grade as the food additive), olive oil, and safflower oil at the ratio of 5:6:1, respectively, to balance the fatty acid composition with Fat-B. The distribution of stearic acid among the acylglycerols was uneven in Fat-B, whereas it was uniform in Fat-C. Because our pre-experiment showed similar results for lipid transport in rats fed stearic

TABLE 3
Fatty Acid Composition, Melting Point, and Iodine Value in Dietary Fats Applied on Experiment 2[a]

	Fat-A (%)	Fat-B (%)	Fat-C (%)
16:0	52.6	10.1	16.1
18:0	4.8	44.8	38.6
18:1	32.1	36.4	37.0
18:2	7.3	6.0	4.9
18:3	0.2	0.5	0.5
Others	3.0	2.2	2.9
Total saturated	57.4	54.9	54.7
Melting point (°C)	46.9	60.0	61.0
Iodine value	42.9	48.6	42.5

[a]Fat-A (dietary fat in which most of the stearic acid was replaced by palmitic acid) was prepared by mixing palm stearin and palm oil (9:1). Fat-B (HSO-rich fat) was prepared with HSO, olive oil, and safflower oil in the ratio 4.5:5:0.5, respectively. Fat-C (monostearoylglycerol-rich fat) contained monoglyceride, olive oil, and safflower oil at the ratio 5:6:1, respectively. In monoglyceride, 72% of component fatty acid was stearic acid and most of the rest was palmitic acid. See Table 1 for abbreviation.

acid-rich interesterified fat and those fed monoacylglycerol-rich fat (data not shown), in this experiment, we adopted monoacylglycerol-rich fat for its easy availability. Another dietary fat (Fat-A), in which most of the stearic acid was replaced by palmitic acid, was prepared by mixing palm stearin (the solid fraction of palm oil, mostly palmitate) and palm oil at the ratio of 9:1 and was used as the comparative fat to examine the stearic acid effects. Palm stearin and palm oil were gifts from Fuji Oil Co. (Osaka, Japan).

Analysis. Blood was centrifuged for 10 min at 1,600 × *g* to obtain plasma. Total cholesterol and high-density lipoprotein (HDL)-cholesterol in plasma were measured using commercially available kits (Cholesterol C-test and HDL-cholesterol C-test; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Liver lipid was extracted by the method of Folch *et al.* (14) and determined gravimetrically. Fecal lipid was extracted by the modified method of Folch *et al.* (14) using an extraction with warm chloroform–methanol solution. In detail, lipid in the freeze-dried feces was extracted by warm Folch solution containing a small amount of water (60°C, 1 h). Reduced to dryness under a N_2 stream, the residue was dissolved in 25 mL petroleum ether over anhydrous Na_2SO_4. Twenty mL of petroleum ether phase was transferred to a tared container, evaporated by N_2 spray and oven-dried (100°C, 1 h). Total lipid was determined gravimetrically. After mild saponification of the lipids, liver cholesterol was extracted and determined by the method of Grundy *et al.* (15). Fecal steroids were extracted by the method of Yamanaka *et al.* (16). Fecal neutral steroids were assayed by the procedure of Grundy *et al.* (15). The concentration of fecal total acidic steroids was assayed by the method of Forman *et al.* (17). The dietary fats and the lipid extracts from feces were methylated at 100°C for 3 h by 5% HCl–methanol. The fatty acid composition of the methylated extracts was measured by a slightly modified procedure of Kawai and Nakayama (18). The triacylglycerol profile in HSO was determined as described by Monsma and Ney (19).

Statistical analysis. Differences among dietary treated groups were assessed by analysis of variance and Duncan's multiple-range test (20). Group means were considered to be significantly different at $P < 0.05$ as determined by the protected least significant difference technique.

RESULT

We investigated the effects of HSO by means of two studies using rats. In Experiment 1, the effects of various concentrations of HSO in the diets with cholesterol on the growth, the cholesterol concentrations of plasma and liver, and the levels of fecal steroids were studied. In Experiment 2, the effects of the distribution of stearic acid in dietary fats, in which each fatty acid was balanced well, on lipid transport in rats were examined.

Experiment 1. As shown in Table 4, the average daily consumption was greater, although body weight gain was lower, among rats fed the HSO50-Chl diet than those on the other diets. Dietary cholesterol did not affect body weight gain and food intake, but it increased liver weight. The effects of dietary HSO and cholesterol on plasma cholesterol, and liver lipid and cholesterol are also shown in Table 4.

Plasma total cholesterol concentration with the control-Chl and HSO25-Chl diets was about 2.5-fold greater than control without cholesterol, whereas it was only 45% greater than control with the HSO50-Chl diet.

The concentration of HDL-cholesterol in rats fed with the HSO50-Chl diet was 74% of that in rats fed with the control diet. However, in rats on the control-Chl or HSO25-Chl diet, it was only 45–56% of that in rats on the control diet. Liver lipid accumulation was about three times greater in rats on the control-Chl and HSO25-Chl diets than in those on the control diet without cholesterol. On the other hand, the accumulation of lipid in the liver induced by dietary cholesterol was only about 75% that of lipid in rats on the HSO50-Chl

TABLE 4
Effect of Highly Hydrogenated, Dietary Soybean Oil with Cholesterol on Body Weight Gain, Food Intake, Relative Liver Weight, Liver Lipids, Liver Cholesterol, Fecal Dry Weight, Fecal Neutral and Acidic Steroids[a]

	Control	Control-Chl	HSO25-Chl	HSO50-Chl
Body wt gain (g/22 d)	97.9 ± 1.57[a]	95.0 ± 5.39[a]	101.0 ± 3.41[a]	80.8 ± 3.99[b]
Food intake (g/22 d)	297 ± 16.0[a]	323 ± 6.8[a]	324 ± 4.1[a]	349 ± 7.8[b]
Relative liver wt (g/100 g b.w.)	3.1 ± 0.11[a]	4.3 ± 0.15[b]	4.2 ± 0.15[b]	4.0 ± 0.16[b]
Total-Chl (mg/dl)	42 ± 4.3[a]	105 ± 6.3[b]	107 ± 6.1[b]	61 ± 4.3[c]
HDL-Chl (mg/dl)	27 ± 2.3[a]	15 ± 2.1[b]	14 ± 2.2[b]	20 ± 2.4[c]
Liver lipids (%)	9.6 ± 0.35[a]	29.3 ± 2.36[b]	27.1 ± 2.41[b]	21.3 ± 1.22[c]
Liver Chl (mg/liver/100 g b.w.)	12.5 ± 1.22[a]	44.1 ± 5.11[b]	40.3 ± 3.91[b]	29.1 ± 1.25[c]
Fecal dry wt (g/day/100 g b.w.)	0.36 ± 0.02[a]	0.41 ± 0.03[a]	0.54 ± 0.03[b]	0.79 ± 0.03[c]
Fecal lipid (%)	4.2 ± 0.92[a]	3.9 ± 0.63[a]	11.3 ± 1.81[b]	32.1 ± 4.54[c]
Fecal cholesterol (mg/day/100 g b.w.)	1.0 ± 0.06[a]	8.7 ± 0.23[b]	14.1 ± 0.83[c]	18.0 ± 0.88[d]
Fecal coprostanol (mg/day/100 g b.w.)	0.8 ± 0.03[a]	2.5 ± 0.26[b]	N.D.	N.D.
Fecal acidic steroids (mg/day/100 g b.w.)	3.75 ± 0.15[a]	7.7 ± 0.46[b]	8.1 ± 0.62[b,c]	8.9 ± 0.55[c]

[a]Each value shows the mean ± SEM of six rats. Values in the same line without common superscript letters denote significant difference ($P < 0.05$). N.D. in the coprostanol line means a value under 0.1 mg/day/100 g body weight (b.w.); HDL, high-density lipoprotein; see Table 1 for other abbreviation. See Table 2 for HSO25 and HSO50 definitions.

diet. The concentration of hepatic cholesterol was 3.2–3.5-fold greater with the control-Chl and HSO25-Chl diets than control, whereas that with the HSO50-Chl diet was only 2.3-fold greater than control.

Table 4 also describes the effects of dietary HSO and cholesterol on the fecal dry weight (g/day/100 g body weight) and the concentration of lipids and steroids in the feces. Fecal dry weight increased with the increased proportion of HSO in the diet. Thus, the feces from animals on the HSO50-Chl diet were twice as heavy as those from rats on the control diet. Accompanying the increase in fecal weight, a marked greater amount of fecal lipids was excreted by rats fed with the HSO50-Chl diet in comparison with those fed with the other diets. Dietary cholesterol appeared not to affect either fecal weight or the concentration of fecal lipids. Excretion of fecal neutral steroids (cholesterol and coprostanol) with the HSO50-Chl diet was twice that with the control-Chl diet. Coprostanol, a metabolite of cholesterol produced in the intestine, was one of the main components in fecal neutral steroids of animals on the control diet. However, this compound was undetectable in feces from animals fed with the diet containing HSO.

Experiment 2. Table 5 shows that feeding Fat-B suppressed body weight gain and promoted food intake, but did not change the relative liver weight compared with Fat-A and Fat-C. Cholesterol supplement did not affect body weight gain or food intake with any dietary fat, but it resulted in a 20% average increase in liver weight. Total plasma cholesterol in rats on the Fat-B diets was significantly less than that on the other diets. Total plasma cholesterol in animals given the Fat-C diet was slightly lower than that on the Fat-A diet, though not significantly. HDL-cholesterol level remained the same among rats on these three diets without supplementary cholesterol. However, the reduction of HDL-cholesterol observed in rats with the Fat-A + Chl and Fat-C + Chl diets was somewhat attenuated in rats with the Fat-B + Chl diet. The amount of liver total lipids in rats fed with the Fat-B diet was significantly less than that with the Fat-A and Fat-C diets, in the absence of added cholesterol. Cholesterol supplement to the Fat-A diet increased the total liver lipids, but this effect was significantly less than that seen on the other two cholesterol-supplemented diets. Liver cholesterol level was similar among rats with these three diets without added dietary cholesterol. The increase in liver cholesterol by dietary cholesterol was greatest in rats given the Fat-A + Chl diet and lowest in rats given the Fat-B + Chl diet. Fecal dry weight was greater in rats on the Fat-B diet than in those on the Fat-A and Fat-C diets, whether or not cholesterol was supplemented. Rats fed with the Fat-B diet excreted greater fecal lipid compared to rats fed the Fat-C or Fat-A diet. The amounts of fecal neutral steroids (cholesterol and coprostanol) were similar among rats fed with the three cholesterol-free diets. However, in cholesterol-containing diets, Fat-B + Chl resulted in a 5-fold increase in neutral steroid excretion compared with a 3.6-fold increase for rats fed Fat-C + Chl ($P < 0.05$). In rats on the Fat-B diet, almost all the fecal neutral steroids consisted of cholesterol, which was different from the pattern of fecal neutral steroids in rats on the other diets, whether cholesterol was supplemented or not. The concentration of fecal acidic steroids was lower on the Fat-A + Chl diet compared with those on the other two diets supplemented with cholesterol. Fatty acid absorption was calculated from the difference between the ingested and excreted amounts of each fatty acid (Table 6). On the Fat-B diet, about 70 and 80% stearic and palmitic acids were absorbed, respectively, regardless of cholesterol supplementation, whereas over 90% of other fatty acids were absorbed. Cholesterol absorption was the lowest in animals given the Fat-B + Chl diet and the highest in those on the Fat-A + Chl diet (Table 6).

DISCUSSION

This study showed that replacing more than 50% of dietary fat by HSO attenuated the rise in plasma cholesterol associated with cholesterol feeding and the HDL-cholesterol reduc-

TABLE 5
Effect of the Distribution of Stearic Acid in Dietary Triacylglycerol on Cholesterol Movement in Rats[a]

	Fat-A	Fat-A + Chl	Fat-B	Fat-B + Chl	Fat-C	Fat-C + Chl
Body wt gain (g/30 d)	133.1 ± 3.35[a]	133.6 ± 2.89[a]	122.2 ± 1.69[b]	121.8 ± 3.04[b]	131.0 ± 1.28[a]	127.1 ± 2.74[a]
Food intake (g/30 d)	394 ± 3.6[a]	398 ± 2.6[a]	420 ±1 3.1[b]	427 ± 7.0[b]	404 ± 6.1[a]	395 ± 7.0[a]
Relative liver wt (g/100 g b.w.)	3.0 ± 0.05[a]	3.9 ±1 0.05[b]	3.2 ±1 0.05[a]	3.8 ± 0.12[b]	3.1 ± 0.07[a]	3.8 ± 0.13[b]
Plasma total cholesterol (mg/dl)	73 ± 10.9[a]	137 ± 11.6[b]	39 ± 3.5[c]	96 ± 4.0[d]	63 ± 11.0[a]	120 ±1 11.6[b]
HDL-cholesterol (mg/dl)	34 ±1 1.1[a]	22 ±1 2.1[b]	36 ± 1.5[a]	29 ± 1.1[c]	33 ± 1.1[a]	24 ±1 1.2[b]
Liver lipid (%)	8.7 ± 0.14[a]	24.4 ±1 0.52[b]	7.2 ± 0.02[d]	20.7 ± 0.23[c]	8.1 ± 0.12[a]	21.9 ± 0.33[c]
Liver cholesterol (mg/liver/100 g b.w.)	5.8 ± 0.28[a]	52.4 ± 2.15[b]	6.4 ± 0.53[a]	40.4 ±12.45[c]	5.7 ± 0.60[a]	46.1 ± 2.42[d]
Fecal dry wt (g/day/100 g b.w.)	0.47 ± 0.031[a,b]	0.45 ± 0.018[a]	0.68 ± 0.019[c]	0.71 ±1 0.040[c]	0.53 ± 0.031[b]	0.47 ± 0.037[a,b]
Fecal lipid (%)	4.7 ± 0.94[a]	3.7 ± 0.30[a]	33.8 ± 2.30[b]	36.5 ± 3.05[b]	8.3 ± 0.27[c]	7.7 ±1 0.32[c]
Fecal cholesterol (mg/day/100 g b.w.)	1.4 ± 0.08[a]	3.7 ±1 0.31[b]	2.2 ± 0.11[c]	11.2 ± 0.61[d]	1.6 ± 0.15[a]	5.9 ± 0.40[e]
Fecal coprostanol (mg/day/100 g b.w.)	1.2 ±" 0.02[a]	2.1 ± 0.10[b]	N.D.	0.3 ± 0.03[c]	0.5 ± 0.01[d]	2.2 ± 0.13[b]
Fecal acidic steroids (mg/day/ g b.w.)	1.5 ± 0.25[a]	3.1 ±1 0.40[b]	1.8 ± 0.31[a]	5.6 ± 0.42[c]	1.6 ±1 0.21[a]	5.4 ± 0.36[c]

[a]Each value shows mean ± SEM of six rats. Values in the same line without common superscript letters denote a significant difference ($P < 0.05$). N.D. in the coprostanol line means a value under 0.1 mg/day/100 g b.w. See Tables 1 and 4 for abbreviations. See Table 3 for Fat-A, Fat-B, and Fat-C definitions.

TABLE 6
The Apparent Absorption of Dietary Fatty Acids and Cholesterol[a]

	Fat-A (%)	Fat-A + Chl (%)	Fat-B (%)	Fat-B + Chl (%)	Fat-C (%)	Fat-C + Chl (%)
16:0	91.0 ± 0.59[a]	94.5 ± 0.22[b]	77.4 ± 1.55[c]	86.8 ± 0.64[d]	95.7 ± 0.24[e]	96.4 ± 0.14[e]
18:0	92.3 ± 0.47[a]	96.8 ± 0.40[b]	69.1 ± 2.21[c]	69.9 ± 1.84[c]	93.0 ± 0.57[a]	92.3 ± 1.49[e]
18:1	98.5 ± 0.10[a]	98.6 ± 0.11[a]	98.2 ± 0.09[b]	99.2 ± 0.09[c]	97.8 ± 0.08[d]	98.2 ± 0.18[b]
Others	96.2 ± 1.06[a]	99.2 ± 0.18[b]	95.7 ± 0.33[a]	97.6 ± 0.21[c]	99.0 ± 0.08[b]	99.8 ± 0.16[c]
Cholesterol		63.1 ± 0.31[a]		52.1 ± 0.31[b]		59.2 ± 0.34[c]

[a]Each value shows the mean ± SEM of six rats. Values in the same line without common superscript letters denote a significant difference ($P < 0.05$). N.D. in the coprostanol line means a value under 0.1 mg/day/100 g b.w. The apparent absorptivities were estimated as the difference between the ingested and excreted amounts of each fatty acid or cholesterol. See Tables 1 and 4 for abbreviations and Table 3 for Fat-A, Fat-B, and Fat-C definitions.

tion induced in rats fed with supplementary cholesterol. The HSO-rich diet also prevented the hepatic lipid and cholesterol accumulation caused by dietary cholesterol in rats. Moreover, the HSO-rich diet increased the fecal mass, fecal lipids, and fecal neutral steroids. There are at least three possible explanations for the alteration of cholesterol transport and metabolism in rats fed with an HSO-rich diet.

The first is based upon the indigestibility and low absorptivity of tristearoylglycerol, the primary component of HSO. Fifty-seven percent of the triacylglycerol composition of HSO was tristearoylglycerol, which is considered to be partially or totally indigestible (21) and not absorbed (12). This low intake of tristearoylglycerol could not be attributed only to its high melting point, because monostearoylglycerol, having a similar or rather higher melting point, is almost completely absorbed. Some other properties, including impaired micelle formation and low susceptibility to lipase, might contribute to poor digestibility of tristearoylglycerol. Regardless, we estimated the absorptivity of stearic acid on the HSO50 diet at 70%. Therefore, the HSO-rich diet could be considered as a low-fat diet. As a low-fat diet improves the lipid profile (22,23), this property of HSO would have significant influence.

The second possibility, which is probably related to the first and may be more relevent, is that HSO, especially the tristearoylglycerol component, would prevent the absorption of dietary cholesterol by a dietary fiber-like action. This possibility is supported by the decreases in body weight gain, plasma cholesterol level and liver cholesterol and by the increases in fecal weight and fecal neutral steroids (Table 4) on the HSO-rich diet compared with those on the control diet, regardless of cholesterol supplementation.

Sugano and his colleagues (24,25) reported several different observations in contrast to our results. They showed that plasma total- and HDL-cholesterol were comparable among four saturated fatty acid-rich diets, including one which was stearic acid-rich, in rats (24) and in hamsters (25). They also showed that there were no differences in body weight gain, food intake, relative liver weight, and fecal weight among animals fed with the experimental diets. The discrepancies between their results and ours are probably due to the different distribution of stearic acid in the dietary triacylglycerols. We used a simple mixture of hydrogenated soybean and natural vegetable oils. This fat contained a higher proportion of tristearoylglycerol than the stearic acid-rich fat used by Imaizumi *et al.* (25), which was prepared by the interesterification of hydrogenated and natural oils.

As described above, the poor absorptivity of tristearoylglycerol might impair intestinal cholesterol absorption. Generally speaking, water-soluble dietary fiber is much more effective than water-insoluble dietary fiber in suppressing plasma cholesterol (26,27). Although HSO is poorly absorbed and has characteristics of water-insoluble dietary fiber, it influences cholesterol transport significantly, like water-soluble dietary fiber. Mechanisms by which soluble dietary fiber reduces plasma cholesterol include suppression of intra-intestinal mobility of cholesterol, reduction of absorptivity by sequestering cholesterol, and the suppression of micelle formation. The mechanism of the dietary fiber-like effect of HSO, probably mainly due to tristearoylglycerol, on cholesterol absorption should be evaluated.

Another possible mechanism for the altered cholesterol transport is the action of post-absorbed stearic acid. In the HSO used in this experiment, 43% of triacylglycerol molecular species consisted of distearoylmonoacylglycerol which is reported to be easily absorbed by the intestine (12). In this study, we found that a significant amount of stearic acid was absorbed (Table 6) especially if fecal stearic acid was considered to be supplemented by a slough of intestinal cells (28), or by intestinal hydrogenation of C-18 unsaturated fatty acid by enterobacteria (9).

Stearic acid has a neutral or hypocholesterolemic effect, which is different from that of other saturated fatty acids (3–6). Bonanome and Grundy (4) have suggested that stearic acid is rapidly desaturated to oleic acid, which seems to be a desirable fatty acid for prevention of arteriosclerosis in humans. Woollett *et al.* (29) have shown that stearic acid did not suppress the rate of receptor-dependent low-density lipoprotein removal from the plasma and/or did not increase the low-density lipoprotein-cholesterol production rate in contrast to other saturated fatty acids in hamsters. Recently, Tholstrup *et al.* (30) reported that fats high in stearic acid may favorably affect the balance between blood coagulation and fibrinolysis in humans, which would induce a less thrombogenic

state by a significant decrease in the level of factor VIIc coagulant activity. Abe *et al.* (24) have reported that stearic acid-rich fat reduced platelet aggregation by ADP and the aortic production of prostacyclin in rats. Thus, stearic acid may contribute to the prevention of arteriosclerosis, but the detailed mechanisms remain controversial.

The ingestion of the HSO-rich diet altered the molecular species of neutral steroids in feces; coprostanol, one of the main neutral steroids in feces from rats on the control diet, was undetectable. As coprostanol is produced by some species of enterobacteria including *Eubacterium* (31), the HSO-rich diet may possibly alter the enterobacteria spectrum. In this study, we did not assay molecular species of acidic steroids in feces, and therefore cannot evaluate the effect of the HSO-rich diets on the enterobacteria spectrum. However, alteration of the enterobacteria spectrum might reduce the quantity of some carcinogenic substances, including sterols and secondary bile acids in the intestine.

In conclusion, dietary HSO reduced the expected increase in plasma and liver cholesterol associated with feeding cholesterol in cholic acid-fed rats. More than half of the stearic acid contained in HSO was tristearoylglycerol, which might play an important role in these effects. However, this triacylglycerol has not been used as a dietary component because of its physical properties and texture. Nevertheless, if food technology overcomes these problems, tristearoylglycerol could contribute to the maintenance of health.

REFERENCES

1. Keys, A., Anderson, J. T., and Grande, F. (1965) Serum Cholesterol Response to Changes in the Diet. IV. Particular Saturated Fatty Acids in the Diet, *Metab., Clin. Exp. 14,* 776–787.
2. Hegsted, D.M., McGandy, R.B., Myers, M.L., and Stare, F.J. (1965) Quantitative Effects of Dietary Fat on Serum Cholesterol in Man, *Am. J. Clin. Nutr. 17,* 281–295.
3. Denke, M.A., and Grundy, S.M. (1991) Comparison Effects of Lauric Acid and Palmitic Acid on Plasma Lipids and Lipoproteins, *Am. J. Clin. Nutr. 54,* 1036–1040.
4. Bonanome, A., and Grundy, S.M. (1988) Effect of Dietary Stearic Acid on Plasma Cholesterol and Lipoprotein Levels, *N. Eng. J. Med. 318,* 1244–1248.
5. Kris-Etherton, P.M., Deer, J., Mitchell, D.C., Mustad, V.A., Russel, M.E., McDonell, E.T., Salabsky, D., and Pearson, T.A. (1993) The Role of Fatty Acid Saturation on Plasma Lipids, Lipoproteins, and Apolipoproteins. I. Effects of Whole Food. Diets High in Cocoa Butter, Olive Oil, Soybean Oil, Dairy Butter, and Milk Chocolate on the Plasma Lipids of Young Men, *Metab., Clin. Exp. 42,* 121–129.
6. Kritchevsky, D., Tepper, S.A., Lioyd, L.M., Davidson, L.M., and Klurfeld, D.M. (1988) Effects of Triglyceride Structure on Lipid Metabolism, *Nutr. Res. 8,* 287–294.
7. Michel, D.C., McMahon, K.E., Shively, C.A., Apgar, J.L., and Kris-Etherton, P.M. (1989) Digestibility of Cocoa Butter and Corn Oil in Human Subjects: A Preliminary Study, *Am. J. Clin. Nutr. 50,* 983–986.
8. Apgar, J.L., Schvely, C.A., and Tarka, S.M. Jr. (1987) Digestibility of Cocoa Butter and Corn Oil and Their Influence on Fatty Acid Distribution in Rats, *J. Nutr. 117,* 660–665.
9. Grigor, M.R., and Dunkley, G.G. (1973) Origin of the High Saturated Fatty Acid Content of Rat Fecal Lipids, *Lipids 8,* 53–55.
10. Hoagland, R., and Snider, G.G. (1943) Digestibility of Certain Higher Saturated Fatty Acids and Triglycerides, *J. Nutr. 26,* 219–225.
11. Cheng, A.L.S., Morehouse, M.G., and Deuel, H.J. Jr. (1949) The Effect of the Level of Dietary Calcium and Magnesium on the Digestibility of Fatty Acids, Simple Triglycerides, and Some Natural and Hydrogenated Fat, *J. Nutr. 37,* 237–250.
12. Mattson, F.H. (1959) The Absorbability of Stearic Acid When Fed as a Simple or Mixed Triglyceride, *J. Nutr. 69,* 338–342.
13. Litchfield, C. (1968) Triglyceride Analysis by Consecutive Liquid-Liquid Partition and Gas Liquid Chromatography, *Ephedra nevadensis* Seed Fat, *Lipids 3,* 170–177.
14. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from AnimalTissues, *J. Biol. Chem. 266,* 497–509.
15. Grundy, S.M., Ahrens, E.H. Jr., and Miettinen, T.A. (1965) Quantitative Isolation and Gas–Liquid Chromatographic Analysis of Total Fecal Bile Acids, *J. Lipid Res. 6,* 397–410.
16. Yamanaka, Y., Tsuji, K., and Ichikawa, T. (1986) Stimulation of Chenodeoxycholic Acid Excretion in Hypercholesterolemic Mice by Dietary Taurine, *J. Nutr. Sci. Vitaminol. 32,* 287–296.
17. Forman, D.T., Phillips, C., Walter, E., and Taylor, C.B. (1968) Fluorometric Measurement of Fecal Bile Acids, *Clin. Chem. 14,* 348–359.
18. Kawai, N., and Nakayama, Y. (1985) Lipids of Fishes I. Lipid Content, Fatty Acid Composition and Cholesterol Content of Teleost Fishes, *Ann. Rep. Osaka City Inst. Pub. Health and Environ. Sci. 48,* 148–164.
19. Monsma, C.C., and Ney, D.M. (1993) Interrelationship of Stearic Acid Content and Triacylglycerol Composition of Lard, Beef Tallow and Cocoa Butter in Rats, *Lipids 28,* 539–547.
20. Duncan, D.B. (1955) Multiple Range and Multiple F Test, *Biometrics 11,* 1–6.
21. Mattil, K.F., and Higgins, J.W. (1945) The Relationship of Glyceride Structure to Fat Digestibility. I. Synthetic Glycerides of Stearic and Oleic Acids, *J. Nutr. 15,* 255–260.
22. Office of Medical Application of Research, National Institute of Health (1985) Lowering Blood Cholesterol to Prevent Heart Disease, *J. Am. Med. Asoc. 253,* 2080–2086.
23. Stein, E.A., Shapero, J., McNerney, C., Glueck, C.J., Tracy, T., and Gartside, P. (1982) Changes in Plasma Lipid and Lipoprotein Fractions After Alteration in Dietary Cholesterol, Polyunsaturated, Saturated, and Total Fat in Free-Living Normal and Hypercholesterolemic Children, *Am. J. Clin. Nutr. 35,* 1375–1390.
24. Abe, K., Imaizumi, K., and Sugano, M. (1993) Effects of Different Triglyceride Saturated Fatty Acids on Tissue Lipid Level, Fatty Acid Composition, Fecal Steroid Excretion, Prostacyclin Production and Platelet Aggregation in Rats, *Biosci. Biotech. Biochem. 57,* 247–252.
25. Imaizumi, K., Abe, K., Kuroda, C., and Sugano, M. (1993) Fat Containing Stearic Acid Increases Fecal Neutral Steroid Excretion and Catabolism of Low Density Lipoproteins Without Affecting Plasma Cholesterol Concentration in Hamsters Fed a Cholesterol-Containing Diet, *J. Nutr. 123,* 1693–1702.
26. Tsuji, E., Tsuji, K., and Suzuki, S. (1975) Effects of Polysaccharides on Cholesterol Metabolism VI. Effects of Various Polysaccharides on Serum and Liver Cholesterol Levels in Cholesterol-Fed Rats, *Jap. J. Nutr. 33,* 273–281.
27. Behall, K.M., Lee, K.H,. and Moser, P.B. (1984) Blood Lipids and Lipoproteins in Adult Men Fed Four Refined Fibers, *Am. J. Clin. Nutr. 39,* 209–214.
28. Shapiro, A., Koster, H., Rittenberg, D., and Schoenhemimer, R. (1936) The Origin of Fecal Fat in Absence of Bile, Studied with Deuterium as an Indicator, *Am., J., Physiol. 117,* 525–528.

29. Woollett, L.A., Spady, D.K., and Dietschy, J.M. (1992) Regulatory Effects of the Saturated Fatty Acids 6:0 Through 18:0 on Hepatic Low Density Lipoprotein Receptor Activity in the Hamster, *J. Clin. Invest. 89,* 1133–1141.
30. Tholstrup, T., Warkmann, P., Jespersen, J., and Sandstrom, B. (1994) Fat High in Stearic Acid Favorably Affects Blood Lipids and Factor VII Coagulant Activity in Composition with Fats High in Palmitic Acid or High in Myristic and Lauric Acids, *Am. J. Clin. Nutr. 59,* 371–377.
31. Mott, G.E., Brinkley, A.W. and Mersinger, C.L. (1980) Biochemical Characterization of Cholesterol–Reducing Eubacterium, *Appl. Environ. Microbiol. 40,* 1017–1022.

[Received October 24, 1994, and in revised form February 23, 1995; Revision accepted April 4, 1995]

Changes in Ether-Linked Phospholipids in Rat Kidney by Dietary α-Linolenic Acid *in vivo*

Jin H. Park, Eek J. Park, Kye S. Kim and Young K. Yeo*

Lipid Chemistry Laboratory, Kyungpook National University, Taegu, 702-701 Korea

ABSTRACT: We investigated the effects of perilla oil containing a high level of α-linolenic acid on *in vivo* phospholipid metabolism, particularly three subclasses of choline glycerophospholipids (CGP) and ethanolamine glycerophospholipids (EGP), in rat kidney. After three weeks of feeding, a significantly lower proportion (by 35%) of the alkylacyl subclass of CGP was found in the perilla oil, as compared to corn oil-fed animals. The alkylacyl species of EGP was also higher in the perilla oil than in the corn oil-fed animals. These alterations were accompanied by a remarkably lower proportion of arachidonic acid and a higher level of eicosapentaenoic acid (EPA) in all six subclasses of CGP and EGP in the perilla oil-fed animals. The levels of linoleic acid were even higher in the diacyl subclasses of CGP and EGP in the perilla oil group, suggesting that desaturase and elongase enzymes prefer n-3 to n-6 fatty acids as substrates for diacyl species. These data are useful in defining the effects of α-linolenic acid on the biosynthesis of renal phospholipids and on the replacement of n-6 with n-3 fatty acids in the six CGP and EGP subclasses.

Lipids 30, 541-546 (1995).

In previous studies, we demonstrated that the biosynthesis of ether-linked forms (1-*O*-alk-1′-enyl-2-acyl and 1-*O*-alkyl-2-acyl subclasses) of choline glycerophospholipids (CGP) in rat kidney *in vivo* was remarkably changed by dietary fish oil enriched in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (1,2). Platelet activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) biosynthesis was markedly reduced in renal tissue in rat by increased intake of fish oil *in vitro* and *ex vivo* (3,4). In addition, very high levels of EPA and DHA accumulated in the ether phospholipids, particularly the ethanolamine plasmalogens, during fish oil consumption, compared to the diacyl phospholipids (5,6). Large proportions of DHA acyl groups were also associated with the ethanolamine plasmalogens when a microsomal membrane fraction isolated from the P388D1 cell was supplemented with n-3 fatty acids (7). It is of interest that ether-containing phospholipids have been proportionally changed by dietary fatty acids although their specific metabolic functions have not been well-elucidated.

α-Linolenic acid, as an n-3 fatty acid, has an effect similar to EPA in terms of *ex vivo* platelet aggregation (8,9) and reduction of serum cholesterol (10). It has also been suggested to prevent thrombosis and myocardial infarction (11), suppress of prostaglandin synthesis (12–14), and alleviate hypertension, although these properties are controversial (15). Perilla oil, which is traditionally a very popular edible oil in oriental countries, contains a high level of α-linolenic acid, up to 65% of the total fatty acids. However, little attention has been devoted thus far to the effects of perilla oil on lipid metabolism in animal tissues.

The purpose of the present work was to evaluate the influence of dietary perilla oil enriched in α-linolenic acid on phospholipid metabolism in rat kidney *in vivo* with emphasis on changes in the ether-linked phospholipids in the CGP and ethanolamine glycerophospholipids (EGP). The distribution of fatty acids in the six CGP and EGP subclasses was also observed.

MATERIALS AND METHODS

Animals and diets. Fourteen female Fischer-344 weaned rats (Korea Chemical Research Institute, Taejeon, Korea), average body weight 35 g, were fed for three weeks *ad libitum* a diet containing either corn oil enriched in linoleic acid (18:2n-6) or perilla oil containing a high level of α-linolenic acid (Table 1). The fatty acid compositions of the corn oil (CO) and perilla oil (PO) diets are shown in Table 2.

Isolation of kidney. The animals were anesthetized with methoxyflurane (Pitman-Moore Ltd., Mississauga, Ontario, Canada). The kidneys from each rat were rapidly removed, decapsulated, perfused with 0.9% saline solution, and weighed. The tissues were homogenized in chloroform/methanol (2:1, vol/vol) using a Polytron homogenizer to obtain total lipid extracts (16).

Lipid analyses. The lipid extracts were separated into various phospholipid classes using thin-layer chromatography (TLC) on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany) developed with chloroform/methanol/acetic acid/water

*To whom correspondence should be addressed.

Abbreviations: CGP, choline glycerophospholipids; CO, corn oil; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EGP, ethanolamine glycerophospholipids; EPA, eicosapentaenoic acid; GPC, *sn*-glycerol-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; PAF, platelet activating factor; PO, perilla oil; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

TABLE 1
Composition of the Corn Oil and Perilla Oil Diets

Ingredients	Composition (wt%)	
	Corn oil	Perilla oil
Casein[a]	20.0	20.0
Choline chloride[b]	0.2	0.2
Cornstarch	12. 5	12. 5
Sucrose	50.0	50.0
Vitamin mix[c]	1.0	1.0
Mineral mix[d]	2.8	2.8
DL-Methionine	0.3	0.3
Cellulose	4.0	4.0
Corn oil[e]	10.0	—
Perilla oil[f]	—	10.0

[a]Namyang Milk Co., Seoul, Korea.
[b]Wako Pure Chemical Industries Ltd., Osaka, Japan.
[c]Provided (μg/g diet): thiamine HCl, 6; riboflavin, 6; pyridoxine HCl, 7; nicotinic acid, 30; DL-calcium pantothenate, 32; folic acid, 2; d-biotin, 0.2; vitamin B-12, 0.1; i-inositol, 1000; vitamin A palmitate, 8; ergocalciferol, 2.5; DL-α-tocopheryl acetate, 37; menadione, 0.5.
[d]Purchased from Taehan Pharmaceutical Co., Seoul, Korea. Provided (per kg): I, 150 mg; Mn, 200 mg; S, 4 g; Co, 100 mg; Fe, 2 g; Zn, 100 mg; Ni, 50 mg; Cu, 100 mg; Mg, 3 g; Ca, 2 g; Se, 40 μg.
[e]Purchased from Jeil Sugar Co., Seoul, Korea.
[f]Extracted from perilla seed.

TABLE 2
Fatty Acid Composition of the Corn Oil (CO) and Perilla Oil (PO) Diets

Fatty acid[a]	Composition (mol%)	
	CO	PO
14:0	0.3	0.2
16:0	12.8	7.4
16:1(iso)[b]	0.3	0.3
18:0	1.8	2.0
18:1n-9	25.1	16.1
18:2n-6	57.8	15.4
18:3n-3	0.8	57.6

[a]Minor fatty acids have been excluded from the table.
[b]iso Means isomers.

(50:37.5:3.5:2, by vol) as a solvent system (17). The phospholipid bands were viewed under ultraviolet light after spraying with 2′,7′-dichlorofluorescein solution (Sigma Chemical, St. Louis, MO) and exposing to ammonia vapor.

In order to separate the 1-*O*-alk-1′-enyl-2-acyl (alkenylacyl), 1-*O*-alkyl-2-acyl (alkylacyl), and 1,2-diacyl (diacyl) CGP and EGP subclasses, the CGP and EGP fractions were incubated with phospholipase C by the method of Bernett *et al.* (18). The resulting diacylglycerols were then acetylated with acetic anhydride and pyridine, and the products were separated by TLC using the developing solvent of petroleum ether/diethyl ether/acetic acid (90:10:1, by vol) followed by a second development in toluene (6). The bands corresponding to the six CGP and EGP subclasses were scraped from the TLC plates and transmethylated with 6% methanolic sulfuric acid in the presence of known amounts of monopentadecanoin (Nu-Chek-Prep, Elysian, MN) as an internal standard to quantitate the acyl chains. The fatty acid methyl esters from the alkenylacyl species were separated from the aldehyde derivatives by TLC using toluene as a solvent. The fatty acid compositions of the various phospholipid classes were analyzed with a Shimadzu GC14A gas chromatograph (Shimadzu Co., Tokyo, Japan) equipped with a DB-225 megabore column (Chromatographic Specialties, Brockville, Ontario, Canada). The flow rate of the nitrogen as a carrier gas was 36 mL/min, and the oven temperature was 210°C isothermal. The molar amounts of total methyl esters derived from the diacyl subclasses were divided by two to compare them quantitatively to the ether lipid subclasses because the acyl groups from the diacyl subclasses are derived from the *sn*-1 and *sn*-2 positions, whereas the acyl groups from the two ether phospholipids represent only the *sn*-2 position. The results were analyzed by a one-way analysis of variance for comparison of the CO and PO groups and expressed as mol%.

RESULTS AND DISCUSSION

There was no significant difference ($P > 0.05$) in body or kidney weights of CO– or PO–fed rats after three weeks of feeding (Table 3). The renal phospholipid composition was very similar for both groups (Table 4). The relative proportions of the six subclasses of renal CGP and EGP in rat are given in Tables 5 and 6, respectively. A significantly lower proportion of alkenylacyl- and alkylacyl-*sn*-glycero-3-phosphocholine (alkenylacyl- and alkylacyl-GPC), relative to diacyl-GPC, was found with dietary perilla oil compared to corn oil (Table 5). The alkenylacyl- and alkylacyl-GPC were remarkably lowered by 65 and 25% in the PO group. The distribution of the alkenylacyl- and alkylacyl-GPC subclasses differed significantly between the two groups, in agreement with the previous reports (1,2) using fish oil. It is of interest that the lower level of alkylacyl-GPC *in vivo* as a precursor of PAF may result in lowering PAF biosynthesis, which is similar to *in vitro* experiments reported by Yeo, Philbrick, and Holub (3). By contrast, the two ether-linked EGP phospholipid subclasses were higher in the PO group by 64 and 57%, respectively, though they are not significantly different (Table 6). The importance of these changes cannot be evaluated until the functions of the ether-linked phospholipids are elucidated in greater detail.

The compositions of the fatty acyl moieties in the three CGP subclasses are provided in Tables 7, 8, and 9. The major unsaturated fatty acyl chain in all three CGP subclasses from the CO group was arachidonic acid, which was 8.8, 5.6, and 10.4 mol% alkenylacyl-, alkylacyl-, and diacyl-GPC, respec-

TABLE 3
Final Body Weight, Kidney Weight and Total Kidney Lipids from Rats Fed Either a Corn Oil (CO) or Perilla Oil (PO) Diet[a]

Parameter	Dietary group	
	CO	PO
Body weight (g)	109.4 ± 2. 9	97. 9 ± 6.1
Kidney weight (g)	1.6 ± 0.1	1.4 ± 0.1
Total kidney lipids (mg/g kidney)	38.7 ± 3.8	45.1 ± 7.8

[a]Values represent means ± SE for seven animals/group.

TABLE 4
Composition of Phospholipids in the Kidneys of Rats Fed Either a Corn Oil (CO) or Perilla Oil (PO) Diet[a]

Phospholipid classes	Composition (μmol/g) CO	PO
CGP	7.3 ± 1.2	8.7 ± 1.5
EGP	6.1 ± 1.5	6.5 ± 0.8
SGP	1.6 ± 0.3	1.9 ± 0.5
SGP	0.8 ± 0.2	0.7 ± 0.2
LPC	0.2 ± 0.02	0.3 ± 0.1
Ratio (CGP/EGP)	1.2/1	1.3/1

[a]Values represent means ± SE for seven animals/group. CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids. SGP, serine glycerophospholipids; IGP, inositol glycerophospholipids; LPC, lysophosphatidylcholine.

TABLE 5
Distribution of the Three Subclasses of CGP in Kidneys of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Phospholipid subclasses	Composition (mol%) CO	PO
Alkenylacyl-GPC	3.6 ± 0.3	2.7 ± 0.3[b]
Alkylacyl-GPC	20.2 ± 3.7	7.1 ± 0.8[b]
Diacyl-GPC	76.2 ± 6.1	90.1 ± 10.8

[a]Values represent means ± SE for seven animals/group. Abbreviations as in Table 4; GPC, *sn*-glycerol-3-phosphocholine.
[b]Indicates significant difference from the CO group at the level of $P < 0.01$.

TABLE 6
Distribution of the Three Subclasses of EGP in Kidneys of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Phospholipid subclasses	Composition (mol%) CO	PO
Alkenylacyl-GPE	17.1 ± 2.30	28.0 ± 8.7
Alkylacyl-GPE	4.7 ± 1.03	7.4 ± 2.4
Diacyl-GPE	78.2 ± 7.40	64.6 ± 7.8

[a]Values represent means ± SE for seven animals/group. Abbreviations as in Tables 4 and 5.

TABLE 7
Acyl Chain Composition of Alkenylacyl-GPC in Kidney Tissues of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Acyl chain	Composition (mol%) CO	PO
14:0	2.5 ± 0.7	4.9 ± 1.6
16:0	33.5 ± 1 7	39.0 ± 4.1
16:1(iso)[b]	3.5 ± 0.2	9.4 ± 4.0
18:0	9.1 ± 1.4	8.5 ± 5.6
18:1(iso)[b]	20.3 ± 4.8	16.8 ± 1.7
18:2n-6	17.0 ± 5.7	5.2 ± 2.7
18:3n-3	0.2 ± 0.2	5.1 ± 1.8[c]
20:3n-6	trace	trace
20:4n-6	8.8 ± 5.8	1.4 ± 0.8
20:3n-3	trace	trace
20:5n-3	0.2 ± 0.1	2.3 ± 0.8
22:4n-6	0.8 ± 0.7	trace
22:5n-6	0.5 ± 0.4	trace
22:5n-3	1.0 ± 0.7	3.1 ± 2.7
22:6n-3	1.5 ± 0.8	3.9 ± 2.5
Uniden	1.1 ± 0.5	0.4 ± 0.1
n-6/n-3	9.3/1	0.5/1

[a]Values represent means ± SE for seven animals/group. Abbreviations as in Table 4.
[b]iso means isomers.
[c]Indicates significant difference from the CO group at the level of $P < 0.05$.

TABLE 8
Acyl Chain Composition of Alkylacyl-GPC in Kidney Tissues of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Acyl chain	Composition (mol%) CO	PO
14:0	2.7 ± 0.1	2.6 ± 0.3
16:0	58.7 ± 6.2	59.4 ± 4.8
16:1(iso)[b]	1.9 ± 0.3	1.4 ± 0.7
18:0	16.9 ± 2.6	18.4 ± 1.9
18:1(iso)[b]	7.6 ± 1.6	6.7 ± 1.1
18:2n-6	0.8 ± 0.8	1.1 ± 0.8
18:3n-3	trace	trace
20:3n-6	trace	trace
20:4n-6	5.6 ± 0.7	2.5 ± 0.9
20:3n-3	trace	trace
20:5n-3	0.4 ± 0.2	1.2 ± 0.6
22:4n-6	trace	trace
22:5n-6	0.4 ± 0.4	1.2 ± 0.7
22:5n-3	3.1 ± 0.4	3.3 ± 0.9
22:6n-3	1.4 ± 0.7	1.6 ± 0.9
Uniden	0.5 ± 0.2	0.6 ± 0.2
n-6/n-3	1.4/1	0.8/1

[a]Values represent means ± SE for seven animals/group. Abbreviations as in Table 5.
[b]iso means isomers.

tively. High levels of arachidonic acid in phospholipids relative to neutral lipids were also reported in other studies (1,19,20,21) when linoleic acid was fed to animals. Animals consuming PO showed remarkably lower levels of the n-6 fatty acids compared to CO animals in all three subclasses of CGP. The proportion of linoleic acid in PO–fed animals was lowered by 69.4% in alkenylacyl-GPC, but was slightly higher in alkylacyl- and diacyl-GPC. It seems that the linoleic acid in the PO group originated mainly from maternal nutrition than from the diet. The different distributions of linoleic acid between dietary PO and fish oil reflect that the affinity of linoleic acid to phospholipids is higher with PO. Much higher levels of α-linolenic acid were shown in the alkenylacyl- and diacyl-GPC subclasses of the PO group, as expected; however, only trace amounts of α-linolenic acid were found in the alkylacyl-GPC of both groups. Arachidonic acid was significantly different between CO and PO group only in diacyl-GPC subclass. All three subclasses of CGP showed much higher levels of EPA in the PO group, with 12, 3, and 27 times higher proportion of alkenylacyl-, alkylacyl-, and diacyl-GPC, respectively. Levels of docosapentaenoic acid (DPA) and DHA were found to be higher in alkenylacyl-GPC than in alkylacyl- or diacyl-GPC in the PO group. Markedly higher levels of the n-3 polyunsaturated fatty acids, such as α-linolenic acid, EPA, and DHA, were exhibited in all CGP classes of the perilla oil group. By contrast, DPA and DHA contributed less to the high levels of n-3 fatty acids in all CGP classes (particularly in alkenylacyl-GPC). The total n-6/n-3 fatty acid ratios for the CO group were 9.3, 1.4, and 11.3 for

TABLE 9
Acyl Chain Composition of Diacyl-GPC in Kidney Tissues of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Acyl chain	Composition (mol%)	
	CO	PO
14:0	0.8 ± 0.03	0.8 ± 0.1
16:0	51.0 ± 0.8	46.0 ± 1.2[c]
16:1(iso)[b]	1.8 ± 0.04	1.9 ± 0.1
18:0	11.5 ± 0.5	14.7 ± 0.4[c]
18:1(iso)[b]	11.1 ± 0.8	10.4 ± 0.6
18:2n-6	9.6 ± 1.03	2.1 ± 1.0
18:3n-3	0.2 ± 0.1	3 0 ± 0.3[c]
20:3n-6	0.6 ± 0.1	0.6 ± 0.3
20:4n-6	10.4 ± 1.04	3.6 ± 0.7[c]
20:3n-3	0.5 ± 0.1	0.8 ± 0.2[c]
20:5n-3	0.1 ± 0.02	2.7 ± 0.3[c]
22:4n-6	0.3 ± 0.02	0.1 ± 0.1[d]
22:5n-6	0.6 ± 0.1	0.2 ± 0.1 [c]
22:5n-3	0.4 ± 0.1	1.0 ± 0.1[c]
22:6n-3	0.7 ± 0.1	1.2 ± 0.1[c]
Uniden	0.4 ± 0 1	0.9 ± 0 3
n-6/n-3	11.3/1	1.9/1

[a]Values represent means ± S.E. for seven animals/group. Abbreviations as in Table 5.
[b]iso Means isomers.
[c]Indicates significant difference from the CO group at the level of $P < 0.01$.
[d]Indicates significant difference from the CO group at the level of $P < 0.05$.

TABLE 10
Acyl Chain Composition of Alkenylacyl-GPE in Kidney Tissues of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Acyl chain	Composition (mol%)	
	CO	PO
14:0	3.8 ± 1.2	4.4 ± 0.7
16:0	31.8 ± 2.6	29.9 ± 3.0
16:1(iso)[b]	2.9 ± 0.9	2.3 ± 0.5
18:0	4.2 ± 0.6	6.7 ± 1.1
18:1(iso)[b]	22.6 ± 2.2	19.1 ± 2.5
18:2n-6	10.1 ± 2.7	7.7 ± 2.7
18:3n-3	trace	3.6 ± 1.4[c]
20:3n-6	trace	0.02 ± 0.01
20:4n-6	19.6 ± 4.1	11.1 ± 2.3
20:3n-3	0.1 ± 0.1	0.2 ± 0.1
20:5n-3	0.1 ± 0.1	8.0 ± 1.7[c]
22:4n-6	1.6 ± 0.5	0.3 ± 0.1 [c]
22:5n-6	0.8 ± 0.3	0.1 ± 0.04[d]
22:5n-3	0.7 ± 0.33	2.5 ± 0.4[c]
22:6n-3	1.3 ± 0.5	3.5 ± 0.4[c]
Uniden	0.4 ± 0.1	0.6 ± 0.2
n-6/n-3	14.6/1	1.1/1

[a,b,c,d]See Table 9 for description.

TABLE 11
Acyl Chain Composition of Alkylacyl-GPE in Kidney Tissues of Rats Fed Either a Corn Oil or Perilla Oil Diet[a]

Acyl chain	Composition (mol%)	
	CO	PO
14:0	3.1 ± 0.5	3.1 ± 2.2
16:0	43.0 ± 7.5	28.3 ± 4.6
16:1(iso)[b]	2.7 ± 0.8	1.8 ± 1.4
18:0	16.0 ± 2.2	1 6.9 ± 3.C
18:1(iso)[b]	10.4 ± 2.1	4.7 ± 2.0
18:2n-6	2.3 ± 2.3	1.6 ± 0.3
18:3n-3	trace	1.8 ± 0.3
20:3n-6	trace	1.1 ± 1.1
20:4n-6	15.1 ± 4.5	1 1.8 ± 2.7
20:3n-3	0.3 ± 0.3	trace
20:5n-3	1.6 ± 1.2	1 2.9 ± 2.0[c]
22:4n-6	1.0 ± 0.5	0.5 ± 0.3
22:5n-6	1.3 ± 0.8	4.6 ± 3.9
2Z:5n-3	1.0 ± 0.6	6.9 ± 1.3[c]
22:6n-3	1.1 ± 0.6	3.6 ± 1.4
Uniden	1.1 ± 0.3	0.4 ± 0.2
n-6/n-3	4.9/1	0.8/1

[a,b,c]See Table 9 for description.

TABLE 12
Acyl Chain Composition of Diacyl-GPE in Kidney Tissues of Rats Fed Either a Corn Oil Or Perilla Oil Diet[a]

Acyl chain	Composition (mol%)	
	CO	PO
14:0	0.1 ± 0.03	3.1 ± 1.4
16:0	15.2 ± 0.6	11.8 ± 0.9[c]
16:1(iso)[b]	1.1 ± 0 04	1.5 ± 0.2
18:0	34.5 ± 0.3	39.6 ± 4.4
18:1(iso)[b]	9.0 ± 0 3	10.4 ± 1.0
18:2n-6	6.4 ± 0.1	7.0 ± 0 9
18:3n-3	0.2 ± 0.04	1.3 ± 0.1 [d]
20:3n-6	0.4 ± 0.1	0.03 ± 0.02[d]
20:4n-6	27.8 ± 0.6	11.6 ± 0.9[d]
20:3n-3	0.6 ± 0.1	1.2 ± 0.6
20:5n-3	0.1 ± 0.03	7.2 ± 1.3[d]
22:4n-6	1.1 ± 0.1	0.6 ± 0.2[c]
22:5n-6	1.7 ± 0.1	0.7 ± 0.2[d]
22:5n-3	0.3 ± 0.1	1.6 ± 0.2[d]
22:6n-3	1.0 ± 0.1	1.5 ± 0.1 [d]
Uniden	0.5 ± 0.2	0.9 ± 0.3
n-6/n-3	17.0/1	1.6/1

[a,b,c,d]See Table 9 for description.

alkenylacyl-, alkylacyl, and diacyl-GPC, respectively. For the PO group, the respective ratios were 0.5, 0.8, and 1.9 for alkenylacyl-, alkylacyl-, and diacyl-GPC. Generally, overall differences in acyl chain compositions of the three subclasses of kidney EGP for the corn oil vs. perilla oil groups indicated trends similar to those found for CGP, although the two diets produced moderate differences in the individual phospholipid subclasses (Tables 10, 11, and 12). The levels of linoleic acid were lower in the alkenylacyl- and alkylacyl-GPE of the PO-fed animals, slightly higher in the diacyl species. Levels of α-linolenic acid were considerably higher in all three subclasses of EGP for the PO group; the CO group had only trace amounts. Levels of arachidonic acid were lowered by 43.4, 21.9, and 58.3% in alkenylacyl-, alkylacyl-, and diacyl-*sn*-glycero-3-phosphoethanol-amine (GPE), respectively, of the PO group. Levels of arachidonic acid with PO feeding were substantially lower in diacyl-GPE subclass, as shown for CGP. A higher proportion of arachidonic acid in the CO group reflected the conversion of linoleic acid to arachidonic acid by enzymic reaction. EPA provided the greatest contribution to the higher levels of n-3 fatty acids in all three subclasses of EGP (8.0 in alkenylacyl-, 12.9 in alkylacyl-, and 7.2 mol% in

diacyl-GPE), which indicated a high conversion rate of α-linolenic acid to EPA by Δ6 and Δ5 desaturase. The proportions of DPA and DHA were 3.6 and 2.7 times higher in alkenylacyl-GPE, 6.9 and 3.3 times higher in alkylacyl-GPE, and 5.3 and 1.5 times higher in diacyl-GPE in the PO–fed animals. All of the n-6 and n-3 fatty acids exhibited pronounced differences between the two dietary groups, whereas only small differences were found in the 16:0, 18:0, and 18:1 acyl chains. The ratios of n-6/n-3 fatty acids in the three subclasses of EGP were around 10 times different between the two dietary groups. The ratios were 14.6 and 1.1 in alkenylacyl-, 4.9 and 0.8 in alkylacyl-, and 17.0 and 1.6 in diacyl–GPE of the CO– vs. PO–fed animals.

This study indicates that ingestion of α-linolenic acid-enriched PO is associated with a reduced proportion of alkylacyl-GPC in rat kidney. The results may bear relevance to previous reports (2–4) on the potential for dietary fish oil to suppress the biosynthesis of PAF. Because the metabolism of the ether-linked phospholipids, particularly alkylacyl-GPC, is closely related to PAF generation and degradation, it is possible that dietary PO may affect PAF levels and metabolism *via* effects on the turnover of the alkylacyl species of CGP. The specific biochemical mechanisms involved in the biosynthesis and turnover of the alkylacyl species of CGP, including those processes specifically documented in the kidney (22–26) and polymornuclear leukocytes (27) that are altered by dietary oil ingestion, remains to be elucidated, although the reactions involved in the turnover of the ether-linked phospholipids in animal tissues have been examined in some detail (22,28–33).

In addition, this study shows that an effect of the PO feeding was a reduced availability of a precursor for the synthesis of the arachidonic acid-derived eicosanoids. The reduction of alkylacyl-GPC synthesis by the replacement of arachidonic acid with α-linolenic acid may also be a potential benefit by reducing the pathophysiological effects of PAF which are mediated by the generation of eicosanoids. The relative availabilities of various n-6 and n-3 fatty acids and their esterified forms, enzyme selectivities, and competition for enzyme binding can regulate their utilization by various *de novo* biosynthetic, deacylation/reacylation, and transacylation reactions leading to CGP and EGP formation in kidney. In general, the diacyl subclasses of CGP and EGP, as major contributors to kidney phospholipids in the PO–fed rats, showed lower amounts of linoleic, arachidonic, adrenic acid, and 22:5n-6, and higher levels of α-linolenic acid, EPA, DPA, and DHA. The fatty acid distribution after dietary α-linolenic acid was somewhat different from that after dietary fish oil, even though both increase n-3 fatty acids. This finding is generally in agreement with the reported replacement of the n-6 by the n-3 fatty acids in kidney tissues of rodents fed fish oil-based diets (34,36) and suggests that the n-3 fatty acids compete with the n-6 fatty acids for the desaturation enzymes; however, both Δ6 and Δ4 desaturases prefer n-3 to n-6 fatty acids as substrates (37). The extent to which dietary PO influences flux through these pathways at the level of substrate selectivity (n-6 vs. n-3 substrates) and enzyme activity or regulation thereof remains to be studied. Further work will be required to determine the effects of replacement of the n-6 by the n-3 fatty acids in the biosynthesis of eicosanoids and PAF as well as the possible effects of these biochemical changes on renal physiology.

ACKNOWLEDGMENTS

This study was supported by a research grant from the Korea Ministry of Education and Woobang Company, Taegu, Korea. We thank Dr. Lloyd A. Horrocks for reviewing the manuscript.

REFERENCES

1. Yeo, Y.K., Philbrick, D.J., and Holub, B.J. (1989) *Biochim. Biophys. Acta 1006*, 9–14.
2. Yeo, Y.K., Philbrick, D.J., and Holub, B.J. (1989) in *Health Effects of Fish and Fish Oils* (Chandra, R.K., ed.) pp. 525–536, ARTS Biomedical Publishers and Distributors Ltd., Newfoundland.
3. Yeo, Y.K., Philbrick, D.J., and Holub, B.J. (1989) *Biochem. Biophys. Res. Commun. 160*, 1238–1242.
4. Sperling, R.I., Robin, J.L., Kylander, K.A., Lee, T.H., Lewis, R.A., and Austen, K.F. (1987) *J. Immunol. 139*, 4186–4191.
5. Aukema, H.M., and Holub, B.J. (1989) *J. Lipid Res. 30*, 59–64.
6. Yeo, Y.K., Philbrick, D.J., and Holub, B.J. (1989) *Biochim. Biophys. Acta 1001*, 25–30.
7. Snyder, F., Blank, M.L., Smith, Z.L., and Cress, E.A. (1991) in *World Rev. Nutr. Diet* (Simopoulos, A.P., Kifer, R.R., Martin, R.E., and Barlow, S.M., eds.) Vol. 66, pp. 383–390, Basel, Karger.
8. Budowski, P., Trostler, N., Lupo, M., Vaisman, N., and Eldor, A. (1984) *Nutr. Res. 4*, 343–346.
9. Ishinaga, M., Kakuta, M., Narita, H., and Kito, M. (1983) *Agric. Biol. Chem. 47*, 903–906.
10. Garg, M.L., Sebokova, E., Wierzbicki, A., Thomson, A.B.R., and Clandinin, M.T. (1988) *Lipids 23*, 847–852.
11. Owren, P.A., Hellem, A.J., and Odegaard, A. (1964) *Lancet 2*, 975–979.
12. Hwang, D.H., and Carroll, A.E. (1980) *Am. J. Clin. Nutr. 33*, 590–598.
13. Adam, O., Wolfram, G., and Zollner, N. (1986) *J. Lipid Res. 27*, 421–426.
14. Bjerve, K.S., Fischer, S., and Alme, K. (1987) *Am. J. Clin. Nutr. 46*, 570–576.
15. Berry, E.M., and Hirsch, J. (1986) *Am. J. Clin. Nutr. 44*, 336–340.
16. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
17. Holub, B.J., and Skeaff, C.M. (1987) *Methods Enzymol. 141*, 234–244.
18. Bernett, J.T., Meredith, N.K., Akins, J.R., and Hannon, W.H. (1985) *J. Liquid Chromatogr. 8*, 1573–1591.
19. Croft, K.D., Beilin, L.J., Vandongen, R., and Mathews, E. (1984) *Biochim. Biophys. Acta 795*, 196–207.
20. Nassar, B.A., Huang, Y.S., and Horrobin, D.F. (1986) *Nutr. Res. 6*, 1397–1409.
21. Yeo, Y.K., and Holub, B.J. (1990) *Lipids 25*, 811–814.
22. Lianos, E.A., and Zanglis, A. (1987) *J. Biol. Chem. 262*, 8990–8993.
23. Schlondorff, D., Goldwasser, P., Neuwirth, R., Satriano, J.A., and Clay, K.L. (1986) *Am. J. Physiol. 250*, 1123–1127.
24. Schlondorff, D., and Neuwirth, R. (1986) *Am. J. Physiol. 251*, 1–11.

25. Schlondorff, D., Goldwasser, P., and Satriano, J.A. (1985) *Clin. Res. 33*, 588A.
26. Chapkin, R.S., and Coble, K.J. (1991) *J. Nutr. Biochem. 2*, 158–164.
27. Horil, T., Satouchi, K., Kobayashi, Y., Saito, K., Watanabe, S., Yoshida, Y., and Okuyama, H. (1991) *J. Immunol. 147*, 1607–1613.
28. Baker, R.C. (1988) *Fed. Am. Soc. Exp. Biol. J. 2*, 6281.
29. Wykle, R.L., Malone, B., and Snyder, F. (1980) *J. Biol. Chem. 2CJ5*, 10256–10260.
30. Woodard, D.S., Lee, T.C., and Snyder, F. (1987) *J. Biol. Chem. 262*, 2520–2527.
31. Blank, M.L., Lee, Y.J., Cress, E.A., and Snyder, F. (1988) *J. Biol. Chem. 263*, 5656–5661.
32. Horrocks, L.A., Yeo, Y.K., Harder, H.W., Mozzi, R., and Goracci, G. (1986) *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Greengard, P. and Robison, G.A., eds.) Vol. 20, pp. 263–285, Raven Press, New York.
33. Renooij, W., and Snyder, F. (1981) *Biochim. Biophys. Acta 63*, 545–556.
34. Swanson, J.E., Black, J.M., and Kinsella, J.E. (1987) *J. Nutr. 117*, 824–832.
35. Schoene, N.W., Ferretti, A., and Fiore, D. (1981) *Lipids 16*, 866–869.
36. Schaap, G.H., Bilo, H.J.G., Popp-Snijders, C., Oe, P.L., Mulder, C., and Donker, A.J.M. (1987) *Life Sci. 41*, 2759–2765.
37. Simopoulos, A.P. (1991) *Am. J. Clin. Nutr. 54*, 438–463.

[Received November 7, 1994, and in revised form March 6, 1995; Revision accepted March 27, 1995]

Inhibition of Gonadotropin-Stimulated Ovarian Steroid Production by Polyunsaturated Fatty Acids in Teleost Fish

François Mercure and Glen Van Der Kraak*

Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1 Canada

ABSTRACT: The effects of the polyunsaturated fatty acids (PUFAs)—eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA)—on *in vitro* steroid production by full-grown prematurational ovarian follicles from goldfish and rainbow trout were investigated. EPA and DHA inhibited gonadotropin-stimulated testosterone production in a dose-related manner, but AA was inhibitory only at the highest dose tested (400 μM). AA alone stimulated testosterone production by increasing cAMP production, but the effects of other PUFAs alone were marginal. The inhibitory actions by PUFAs were not restricted to long-chain PUFAs, as linoleic and linolenic acids had similar actions in the goldfish. The inhibitory action of EPA on testosterone production was reversible upon removal of the PUFA from medium. Testosterone production stimulated by the addition of the cAMP analogues, dibutyryl cAMP, and 8-bromo cAMP, was attenuated by PUFAs, suggesting that they act at a site distal to cAMP formation. A post-cAMP site regulating cholesterol availability may be involved as testosterone production induced by addition of 25OH-cholesterol was not affected by the PUFAs in either fish species. Together, these findings underscore the importance of lipids in ovarian physiology and suggest that PUFAs may participate in the regulation of ovarian steroidogenesis in teleost fish.
Lipids 30, 547–554 (1995).

Environmental influences on long-chain polyunsaturated fatty acids' (PUFAs) composition and metabolism in fish have been considered by many (1–4). However, even though lipid composition in fish is seasonal and may be related to reproductive status (5), little is known of how PUFAs affect ovarian physiology. In freshwater fish, arachidonic acid (AA) (20:4n-6) is usually the most abundant tetraenoic fatty acid, eicosapentaenoic acid (EPA) (C20:5n-3) is often the predominant pentaenoic acid, and docosahexaenoic acid (DHA) (C22:6n-3) is, without exception, the main hexaenoic acid (5). The physiological significance of AA as an eicosanoid precursor and in signal transduction is well established (6–9), as is the importance of eicosanoids, such as prostaglandins, as regulators of ovarian physiology in fish and in many vertebrates (10). However, the roles of EPA and DHA in fish and in mammals remain relatively unclear, even though they are the focus of much research in human health (11,12).

In reproduction, lipids are most often considered from an energetic point of view, and their possible involvement in other physiological processes are often overlooked. Selective lipid depletion has been observed in spawning salmonids (13), and selective PUFA utilization has been reported during gonadal development of salmonids and goldfish (14–16). AA, EPA, and DHA were preferentially released from arctic char ovarian follicles in the presence of melittin, a phospholipase A_2 activator (Geritts, M., Van Der Kraak, G., and Ballantyne, J., unpublished data), and from goldfish testicular fragments upon stimulation with a protein kinase C activator and a calcium ionophore (17). AA, *via* its conversion to eicosanoids, stimulates testosterone production in goldfish ovaries (18), and also in testes (19). By comparison, EPA and DHA inhibit gonadotropin-stimulated testosterone production by goldfish testis (17). PUFAs also modulate gonadal steroidogenesis in avian and in mammalian species (20–25).

This study examines the effects of unesterified long-chain PUFAs including AA, EPA, and DHA on goldfish and rainbow trout ovarian steroidogenesis *in vitro*. Testosterone production was used as an index of steroidogenic activity, as it is a major steroid secreted by full-grown, prematurational ovarian follicles of teleost fish (26,27).

MATERIALS AND METHODS

Fish. Goldfish (*Carassius auratus*), common or comet varieties, were purchased from Grassyfork Fisheries Co. (Martinsville, IN). Fish were held in 1.2-m diameter tanks with flow-through water at 15–18°C under a constant photoperiod (14 light/10 dark). Fish were fed a commercial trout diet once daily to satiation. Prespawning female rainbow trout (*Oncorhynchus mykiss*) were obtained from the Alma Aquaculture Research Station (Alma, Ontario, Canada). Trout were held at the University of Guelph for a period no longer than a week, at 8–11°C.

* To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; 25-OH Chol, 25-hydroxycholesterol; dbcAMP, dibutyryl cAMP; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; hCG, human chorionic gonadotropin; IBMX, 3-isobutyl-1-methylxanthine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PUFA, polyunsaturated fatty acid; SG-G100, partially purified salmon gonadotropin.

Copyright © 1995 by AOCS Press

Ovarian follicle incubations. The follicle incubations followed the general methods described by Van Der Kraak and Chang (18). Fish were sacrificed by spinal transection, and ovaries were immediately placed in a modified Cortland's medium consisting of (g/L): NaCl, 7.25; KCl, 0.38; $CaCl_2 \cdot 2H_2O$, 0.23; $NaH_2PO_4 \cdot H_2O$, 0.41; $MgCl_2 \cdot 6H_2O$, 0.20; $MgSO_4 \cdot 7H_2O$, 0.23; $NaHCO_3$, 1.0; bovine serum albumin, 1.0; glucose, 1.0; and streptomycin sulfate, 0.1; pH 7.6. Each experiment used full-grown, prematurational follicles from a single fish. Due to the asynchronous development of goldfish follicles, full-grown follicles (0.9–1.1 mm in diameter) were manually sorted from the smaller vitellogenic follicles using a Pasteur pipette before placing them in polystyrene (24-well) tissue culture plates (Falcon 3047; Toronto, Ontario, Canada) containing incubation medium. Each treatment consisted of four replicate incubations of 20 follicles for goldfish or 10 full-grown follicles (4–6 mm in diameter) for rainbow trout. Immediately prior to the addition of test chemicals and/or hormones, the medium was replaced with fresh medium. Goldfish follicles incubations routinely included the phosphodiesterase inhibitor 3isobutyl-1-methylxanthine (IBMX) at 1 mM to ensure detectable basal steroid levels and to facilitate measurement of cAMP. IBMX was not added to trout follicle incubations, as basal levels were often higher in this species. Treatment chemicals and hormones were added from concentrated stocks and diluted to working concentrations in the incubation medium. Final incubation volume was 1 mL. Fresh aliquots of fatty acids were opened immediately prior to each experiment to ensure their integrity. Incubations were in air at 18°C in the dark. Incubation periods were routinely for 18 h or as indicated in the figure legends. Following incubation, 0.8 mL of medium was removed from each well and placed in separate borosilicate test tubes to be stored at –20°C for subsequent radioimmunoassay. Media collected for cAMP measurement was boiled immediately for 15 min to destroy phosphodiesterase activity prior to freezing.

The protocols for the testosterone and cAMP assays were described by Van Der Kraak and Chang (18), and by Van Der Kraak (28), respectively. Medium from each replicate was assayed in duplicate.

Chemicals and hormones. Unesterified free fatty acids, human chorionic gonadotropin (hCG), forskolin, IBMX, dibutyryl cAMP (dbcAMP), 8-bromo cAMP, and 25-hydroxycholesterol (25-OH Chol) were obtained from Sigma Chemical Co. (St. Louis, MO). Partially purified salmon gonadotropin (SG-G100) was a gift from Dr. E.M. Donaldson (Department of Fisheries and Oceans, West Vancouver, British Columbia, Canada). hCG, SG-G100, IBMX, and dbcAMP were dissolved directly in incubation medium; and forskolin, free fatty acids, and 25-OH Chol were dissolved in absolute ethanol. 8-Bromo cAMP was solubilized in dimethyl sulfoxide (DMSO). The final volume of ethanol never exceeded 1.0% of final incubation volume, and this concentration has no effect on basal or gonadotropin-stimulated testosterone production (18). Similarly, DMSO at final concentration of 0.5% had no effect on steroid production (data not shown).

Statistical analysis. Following logarithmic transformation (Log_{10}), differences between means were assessed by one-way analysis of variance and by Duncan's Multiple Range test. A P value of ≤ 0.05 was considered significant. Values reported in all figures represent the mean ± SEM of four replicate incubations, and representations lacking error bars indicate a smaller error than the symbol depicting the mean. Data presented are representative of at least two experiments yielding similar results.

RESULTS

Effects of PUFAs on steroidogenesis. Experiments were conducted to test the effects of graded dosages of PUFAs (6.25–400 µM) on ovarian steroidogenesis of goldfish and rainbow trout. Consistent with earlier studies, hCG caused a marked increase in testosterone production by goldfish prematurational follicles (Fig. 1). Addition of EPA and DHA caused similar inhibitions of hCG-stimulated testosterone production, with the effects being significant at the higher doses (100–400 µM). AA was also inhibitory, but only at the highest dose tested. AA alone stimulated testosterone production in a dose-dependent manner with maximum stimulation achieved at 100 µM. EPA and DHA caused marginal increase in testosterone production at the higher dosages. In the trout, DHA inhibited SG-G100-stimulated testosterone production in a dose-related manner, but the inhibition by AA was significant only at the highest dose (Fig. 2). AA at 6.25 µM, but not at higher dosages, stimulated testosterone production.

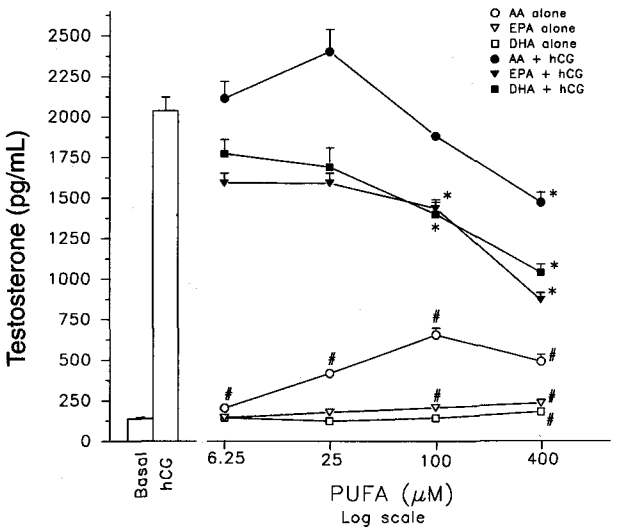


FIG. 1. Effects of graded dosages of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) on basal- and on human chorionic gonadotropin (hCG)-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. * Indicates a significant difference from hCG alone, and # indicates a significant difference from basal level.

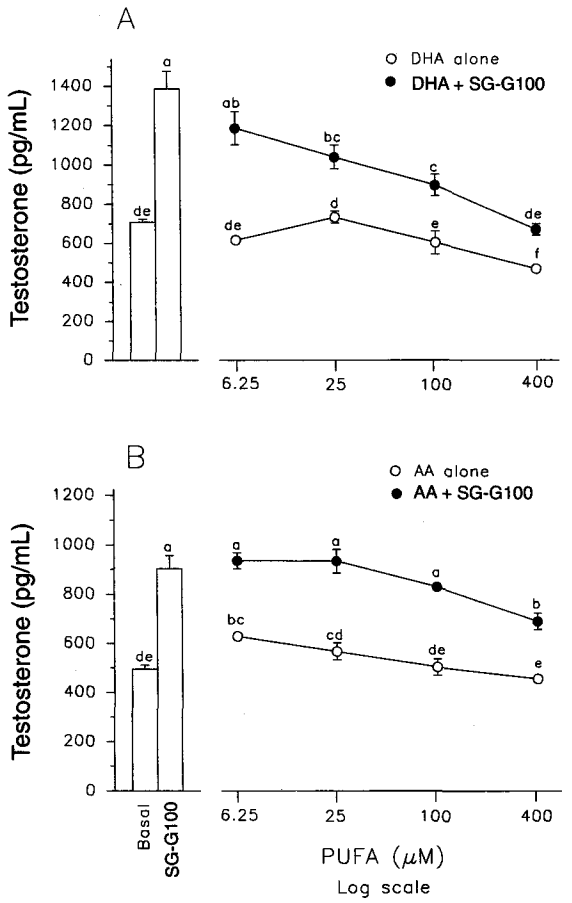


FIG. 2. Effects of graded dosages of DHA (A) and AA (B) on basal- and on partially purified salmon gonadotropin (SG-G100) (1 μg/mL) stimulated testosterone production by rainbow trout full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

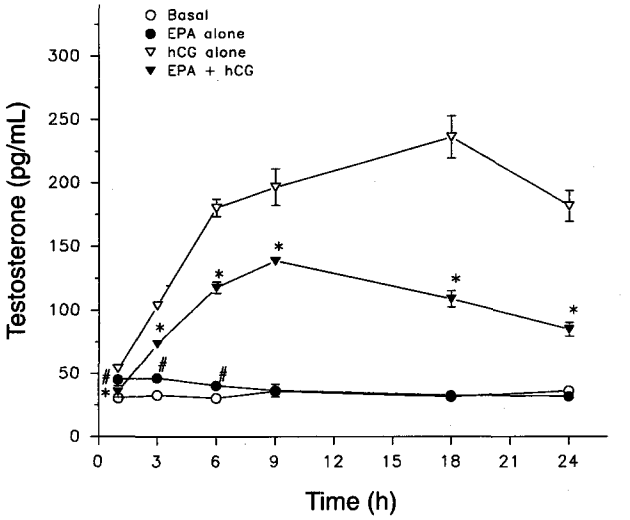


FIG. 3. Time course response of EPA (400 μM) effects on basal- and hCG-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated at 18°C. Values represent the mean ± SEM of four replicate incubations. * Indicates a significant inhibition by EPA from hCG alone, and # indicates a significant difference from basal level at each time interval. Abbreviations as in Figure 1.

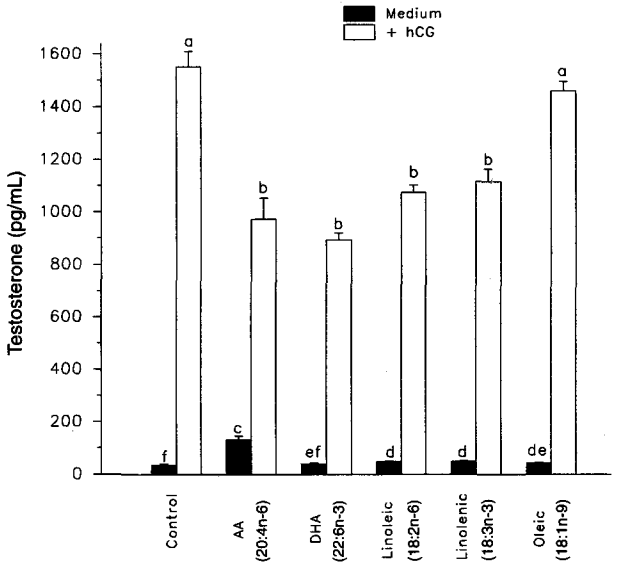


FIG. 4. Effects of AA, EPA, linoleic acid, linolenic acid, and oleic acid at 400 μM on basal- and on hCG-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

EPA was found to have rapid effects in time course experiments with goldfish follicles (Fig. 3). EPA attenuated hCG actions, but increased basal testosterone production after the first hour on its own. Maximal stimulation by hCG was found after 18 h, where inhibition by EPA was also greatest. The initial stimulation of testosterone production by EPA alone subsequently disappeared after six hours of incubation.

Experiments were conducted to test the specificity of the effects found with the long-chain PUFAs. In addition to AA and DHA, linoleic, and linolenic acids at 400 μM attenuated hCG-stimulated testosterone production by goldfish ovarian follicles, although oleic acid had no significant effect (Fig. 4). Basally, AA, linoleic, linolenic, and oleic acids had stimulatory effects, with AA being most effective.

Effects of PUFA removal. Given that relatively high dosages of fatty acids were routinely used to inhibit gonadotropin-stimulated testosterone production, experiments were conducted with goldfish to establish whether the decrease in steroidogenesis was due to effects on follicular viability. The approach was to conduct an initial 15-h incubation testing the actions of graded dosages of EPA and, following removal of EPA by washing several times with fresh medium, to measure the follicular response to hCG during a subsequent 15-h incubation. Media were removed and frozen until assayed for testosterone after each 15-h incubation. In initial in-

cubation, follicles responded with the typical stimulation by hCG, and EPA attenuated hCG actions (Fig. 5A). In the subsequent incubation, basal testosterone production was maintained: 63 and 64 pg/mL in initial and in subsequent incubations, respectively. However, the overall responsiveness of follicles to hCG was much less than in initial incubation (Fig 5B). Most importantly, the follicles initially exposed to hCG alone and hCG in combination with EPA were equally responsive during a second incubation with hCG. These data demonstrate that although responsiveness of follicles to hCG was affected after 15 h of incubation, the inhibition by EPA was reversible.

Sites of inhibition. Because gonadotropin stimulates steroid production *via* cAMP, the effects of PUFAs on cAMP and testosterone production were assessed in goldfish. As expected, addition of hCG increased accumulation of cAMP in the media (Fig. 6). Addition of EPA (100 and 400 μM) inhibited hCG-stimulated cAMP production (Fig. 6A). EPA alone caused a slight but significant dose-related accumulation of cAMP in the media, but this effect did not match the effect on testosterone production, as EPA at 100 μM was inhibitory, whereas EPA at 400 μM was stimulatory. AA alone increased cAMP levels in a dose-related manner which corresponded with an increase in testosterone production (Fig. 6B). However, AA potentiated hCG-stimulated cAMP production but did not affect testosterone production. Relatively low levels of testosterone were produced in these experiments compared to previous results, due to the shorter (4-h) incubation period used for cAMP assessment.

Phosphodiesterase activity is an important intracellular regulatory site controlling cAMP levels. Because the phosphodiesterase inhibitor IBMX was necessary to detect cAMP levels, and because it was routinely added to all goldfish follicle incubations, it was important to test whether IBMX affected the actions of the PUFAs. IBMX at dosages of 0.1 and 1.0 mM led to a modest increase in basal testosterone production, but markedly enhanced the stimulatory actions of hCG (Fig. 7). More importantly, EPA attenuated hCG-stimulated testosterone production both in the presence or absence of IBMX.

A series of experiments were conducted to test whether PUFAs may act at other sites post-cAMP formation. The

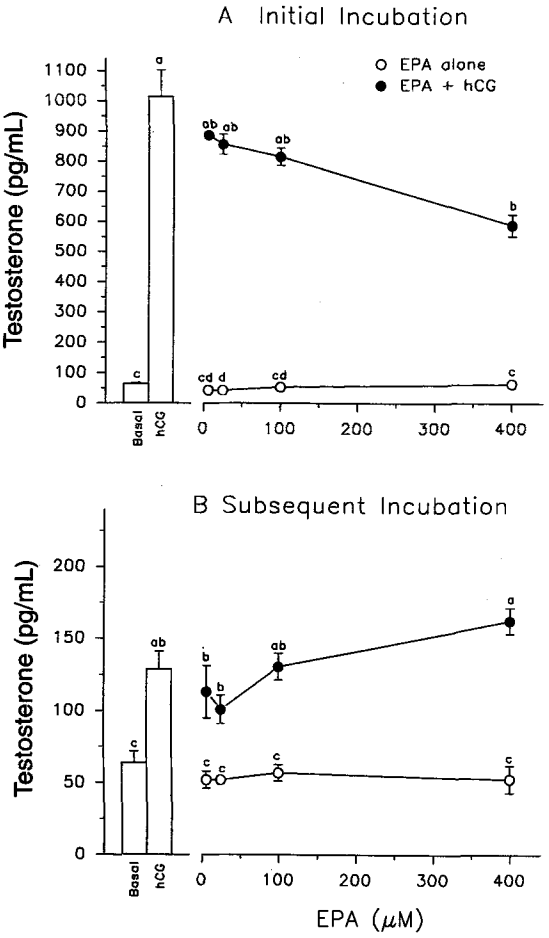


FIG. 5. Assessment of follicular responsiveness to hCG following the removal of EPA. Effects of graded dosages of EPA on basal- and hCG-stimulated testosterone production by goldfish full-grown ovarian follicles after a 15-h initial incubation at 18°C (A). Basal- and hCG-stimulated testosterone production of the same follicles during a subsequent 15-h incubation after removal of EPA and in fresh medium with and without hCG (B). Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

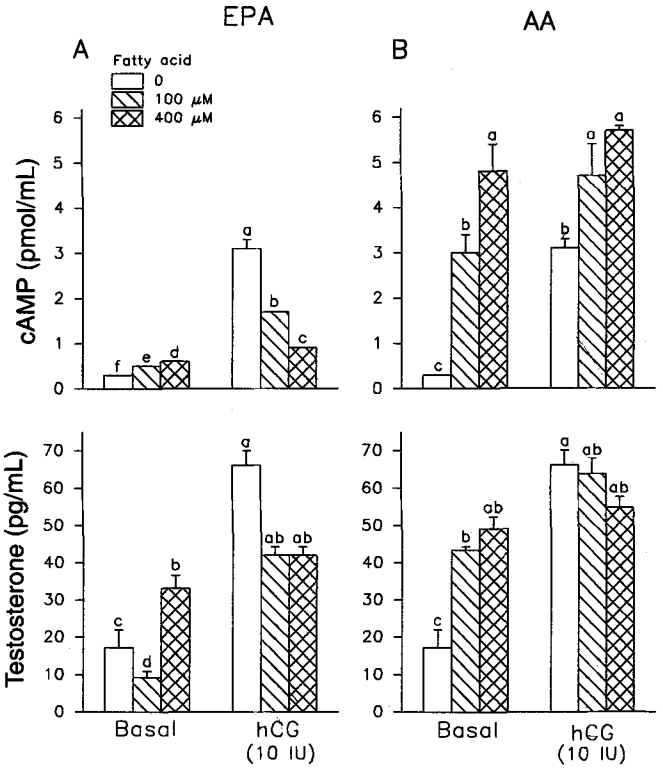


FIG. 6. Effects of EPA (A) and AA (B) on basal- and hCG-stimulated cAMP and testosterone production by goldfish full-grown ovarian follicles incubated for 4 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

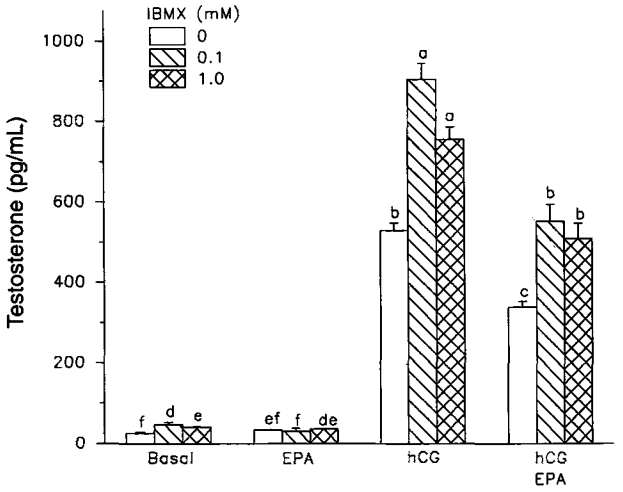


FIG. 7. Dosage effects of 3-isobutyl-1-methylxanthine (IBMX) on basal- and hCG-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated for 18 h at 18°C with and without EPA (400 μM). Values represent the mean ± SEM of four replicate incubations. Means with different subscripts are significantly different. Abbreviations as in Figure 1.

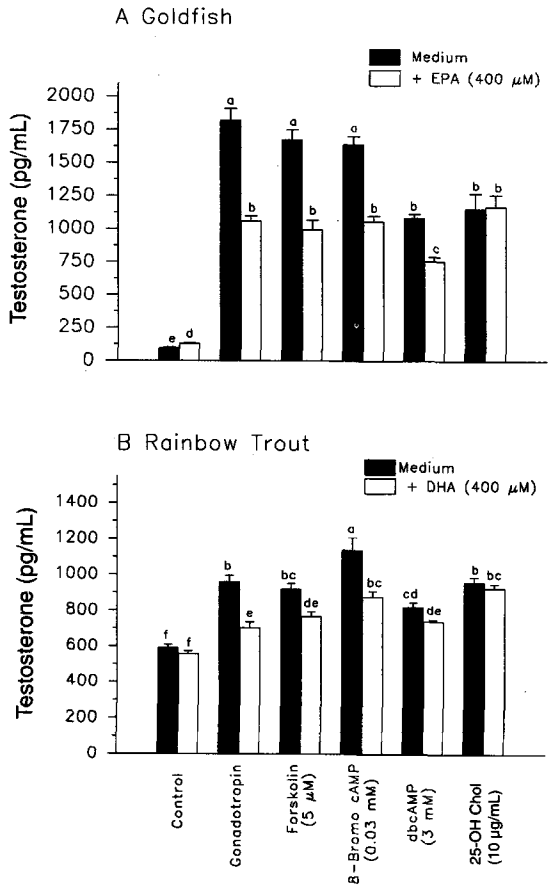


FIG. 8. Effects of polyunsaturated fatty acids on hCG (SG-G100 for trout), forskolin, 8-Bromo cAMP, dibutyryl cAMP (dbcAMP), and 25-OH Cholesterol (25-OH chol) stimulated testosterone production by goldfish (A) and rainbow trout (B) full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figures 1 and 2.

stimulatory actions of hCG and forskolin, a direct activator of adenylate cyclase, on testosterone production were inhibited by EPA in the goldfish (Fig. 8A). Testosterone production induced by two cAMP analogues, 8-Bromo cAMP and dbcAMP, which mimic the actions of cAMP, were also inhibited by EPA, indicating an effect distal to cAMP formation. When follicular testosterone production was stimulated by addition of the steroidogenic substrate 25-OH Chol, EPA was without effect. In the rainbow trout, SG-G100, forskolin, and 8-Bromo cAMP actions on testosterone production were inhibited by the addition of DHA (Fig. 8B). However, it is not clear why DHA did not block the dbcAMP-induced increase in testosterone production in the trout. As with the goldfish, the stimulatory effect of 25-OH Chol was not affected by DHA.

DISCUSSION

These studies demonstrate differential actions of PUFAs on basal and on gonadotropin-stimulated steroid production in goldfish and in rainbow trout ovarian follicles. In both species, PUFAs inhibit the stimulatory action of gonadotropin at a site distal to cAMP formation which may regulate cholesterol supply to the steroidogenic pathway. AA alone stimulated testosterone production by increasing cAMP production, but the effects of other PUFAs alone were marginal. These studies support the contention that unesterified long-chain PUFAs, including AA, EPA, and DHA, participate in the regulation of ovarian steroidgenesis in teleosts.

Gonadotropin stimulation of steroidogenesis in teleosts involves the cAMP signal transduction pathway (28–31), and studies with mammals have established that cAMP plays a role in regulating the synthesis, storage, and transport of cholesterol in steroidogenic tissues (32–36). EPA inhibited gonadotropin actions on steroid production by blocking cAMP formation. Given that the phosphodiesterase inhibitor IBMX was added to the incubation medium, the effect of EPA was on cAMP formation rather than on its degradation. However, AA at high dosage inhibited gonadotropin-stimulated testosterone production in spite of its potentiation on cAMP levels, suggesting a second site of action distal to cAMP. This was confirmed in studies showing that PUFAs also inhibited testosterone production stimulated by addition of cAMP analogues. This distal site must affect substrate availability to the steroidogenic enzymes as the PUFAs did not inhibit testosterone production stimulated by 25-OH Chol. It is becoming evident that regulation of substrate availability may be central in controlling gonadal steroidogenesis, as the inhibitory actions of other messengers, such as growth factors and cytokines, on steroid production were also reversible upon addition of exogenous cholesterol (37–39).

As with previous studies with goldfish ovarian follicles (18,28), there was variation in the basal, and gonadotropin-stimulated testosterone production. Although several factors, such as follicular maturity, may affect responsiveness to gonadotropin and steroid production during the preovulatory pe-

riod, PUFAs consistently inhibited gonadotropin-stimulated steroid production. Similarly to vitellogenic goldfish follicles, AA, EPA, and DHA attenuated hCG-stimulated testosterone and 17ß-estradiol production (McFarlane, W., Mercure, F., and Van Der Kraak, G., unpublished).

Because PUFAs can inhibit gonadotropin-stimulated testosterone production independently of their effects on cAMP formation or follicular maturity, intracellular mechanisms other than the cAMP signal transduction pathway may be involved. Several other transduction pathways participate in the regulation of ovarian function in mammals (40,41) and in teleost species (28,42–46). Stimulation of the lipid and calcium dependent phosphoinositol (PI) transduction pathway, with a tumor promoting phorbol ester (phorbol 12-myristate 13-acetate, PMA) and calcium ionophore A23187, was inhibitory to testosterone production in goldfish ovarian follicles (18,45). PMA is known to activate protein kinase C (PKC) (47,48), and in mammalian species PUFAs directly modulate PKC activity (23,24). Therefore, the inhibitory action of PUFAs on testosterone production in teleosts could be mediated by PKC activation. However, in goldfish testicular tissue, in which PUFAs inhibited hCG-stimulated testosterone production, PUFAs failed to activate PKC, suggesting some differences between mammals and fish (49). In chicken ovarian tissue, AA, which also inhibited gonadotropin-stimulated steroid production, failed to activate PKC (21,22). The inhibitory action of AA in hen ovarian granulosa cells appeared to be calcium-dependent (22). The inhibition of steroidogenesis in mouse testicular Leydig cells by nonesterified fatty acids occurs at a step prior to the conversion of cholesterol which was also found to be calcium-dependent (20). Thus, it is plausible that the inhibitory action of PUFAs in fish ovaries may be mediated by a calcium-dependent mechanism. In earlier studies, high dosages of A23187, which would elevate intracellular calcium levels, inhibited gonadotropin-stimulated testosterone production by goldfish prematurational ovarian follicles (28,43).

Since the inhibition of gonadotropin-stimulated testosterone production by PUFAs was not specific to any fatty acid and was found in both species studied, it is unlikely that specific metabolites are responsible for the inhibition. Thus, the inhibitory effect could have been the consequence of some shift in lipid and cholesterol metabolism induced by the PUFAs. Cholesterol metabolism is intimately linked with lipid absorption, transport, and metabolism; and in poikilotherms such as fish, regulation of lipid uptake, membrane integrity, and metabolic pathways must be integrated (50). Recent findings suggest that PUFAs act in a hormone-like manner to regulate the expression of peroxisomal enzymes involved in the metabolism of cholesterol and of long-chain PUFAs (51–56). Peroxisome proliferators are chemicals which induce peroxisomal enzymes by disrupting lipid metabolism, resulting in a fatty acid overload responsible for the induction (57–59). Given the relatively high doses tested and the nonspecificity of the PUFA inhibitory action in this study, it is possible that PUFAs inhibit by way of peroxisomal induction. Preliminary results have shown that recognized peroxisome proliferators inhibit ovarian steroid production of goldfish follicles (Mercure, F., and Van Der Kraak, G., unpublished). Thus, the fatty acid overload model proposed by Lock *et al.* (57), whereby PUFAs induce metabolic changes, warrants more attention in the present experimental model.

The paradoxical effects of AA on basal- and gonadotropin-stimulated testosterone production may relate to differential effects of AA and its metabolites. The finding that AA alone stimulates ovarian steroid production in goldfish and trout was consistent with previous studies showing that the actions of AA were dependent on its conversion to cyclooxygenase metabolites in goldfish ovaries (18) and testes (19), and that prostaglandins stimulate testosterone production *via* cAMP in goldfish testis (19) and in ovaries (Mercure, F., and Van Der Kraak, G., unpublished). However, there were differences in the sensitivity to AA alone between goldfish and trout. Given that temperature affects membrane lipid composition (1–4) and eicosanoid production in the goldfish (Mercure, F., and Van Der Kraak, G., unpublished), important differences may exist with respect to PUFA conversion to eicosanoids in goldfish and trout, which are warm and cold water species, respectively. Although EPA and DHA also affect basal steroid production, these data should be interpreted with caution, as stimulation was modest and not always statistically significant.

We were concerned that the high dose of fatty acids routinely used in this study caused cytotoxicity resulting in lower steroid production. This study demonstrated that PUFAs even at the highest dose did not affect follicular viability, as the inhibition was reversible upon removal of the PUFAs. However, it is unclear why the overall follicular responsiveness to hCG was greatly reduced after 15 h of incubation, and over time. It is likely that the overall follicular steroidogenic capacity diminishes with time under the incubation conditions used. Expression of steroidogenic enzymes, and thus their activity, was found to decline with time in similar incubations of rainbow trout and medaka ovarian follicles (Tanaka, M. and Nagahama, Y., personal communication). Nevertheless, this study demonstrates that PUFAs do not irreversibly affect steroidogenesis, which is consistent with the findings of others. Meikle *et al.* (20) reported, using trypan blue exclusion and chromium-51 release tests, that unesterified fatty acid concentrations up to 2,900 μM had no effect on membrane integrity and cell viability of mouse Leydig cells. Further, they demonstrated that the inhibition by nonesterified fatty acids, such as oleic and linoleic, was reversible upon removal of the fatty acids.

Although the high doses of PUFAs added were not lethal to the cells, the physiological significance of such doses must be addressed. The levels of nonesterified plasma fatty acids in fish are often much higher than in mammals, and large concentrations of nonesterified PUFAs have been reported in the gonads of some fish (5). Plasma levels ranging between 1,200 and 18,500 μM of total nonesterified fatty acids have been measured for several fish species (60), which is significantly

higher than the experimental dosages tested in the present study. As a result, the dosages tested may be regarded as physiological in fish. Due to the hydrophobicity of the fatty acids, dosages in excess of 400 μM could not be tested under the conditions used.

In summary, PUFAs have been found to inhibit gonadotropin-stimulated steroidogenesis in a cold and a warm water fish species. PUFAs have varying effects on cAMP formation, but apparently act in a similar manner at a site distal to cAMP to inhibit testosterone production. Their inhibitory actions were reversible. Intracellular transduction mechanisms other than the cAMP signal transduction pathway, possibly a calcium-dependent mechanism, may mediate the action of the PUFAs. Finally, PUFAs may induce changes in cellular metabolism with consequences on steroidogenic activity. These results implicate PUFAs as putative regulators of ovarian function in teleost fish.

ACKNOWLEDGMENTS

This work was supported by Operating Grant U0554 from the Natural Sciences and Engineering Research Council of Canada to G.V.D.K. The rainbow trout used in this study were donated by the Alma Aquaculture Research Station of the University of Guelph and the Ontario Ministry of Agriculture and Food. The antisera for testosterone and cAMP were generously donated by Dr. R.J. Etches (University of Guelph) and Dr. J.P. Chang (University of Alberta), respectively. SG-G100 was generously provided by Dr. E.M. Donaldson (Department of Fisheries and Oceans, West Vancouver, British Columbia, Canada).

REFERENCES

1. Hazel, J.R. (1984) *Am. J. Physiol. 246*, R460–R470.
2. Bell, M.V., Henderson, R.J., and Sargent, J.R. (1986) *Comp. Biochem. Physiol. 83B*, 711–719.
3. Tocher, D.R., and Sargent, J.R. (1990) *Lipids 25*, 435–442.
4. Hazel, J.R., and Williams, E.E. (1990) *Prog. Lipid Res. 29*, 167–227.
5. Henderson, R.J., and Tocher, D.R. (1987) *Prog. Lipid Res. 26*, 281–347.
6. Downes, C.B., and Michell, R.H. (1985) in *Molecular Mechanisms of Transmembrane Signalling* (Cohen, P., and Houslay, M.D., eds.) pp. 3–56, Elsevier, New York.
7. Rasmussen, H., Apfeldorf, W., Barrett, P., Takuwa, N., Zawalich, W., Kreutter, D., Park, S., and Takuwa, Y. (1986) in *Phosphoinositides and Receptor Mechanisms* (Putney, J.W., ed.) pp. 109–148, Alan R. Liss Inc., New York.
8. Smith, W.L. (1989) *Biochem. J. 259*, 315–324.
9. Naor, Z. (1991) *Mol. Cell. Endocrinol. 80*, C181–C186.
10. Murdoch, W.J., Hansen, T.R., and McPherson, L.A. (1993) *Prostaglandins 46*, 85–115.
11. Simopoulos, A.P., Kifer, R.R., and Martin, R.E. (1986) *Health Effects of Polyunsaturated Fatty Acids in Seafoods*, Academic Press, New York.
12. Lees, R.S., and Karel, M. (1990) *Omega-3 Fatty Acids in Health and Disease*, Marcel Dekker, Inc., New York.
13. Phleger, C.F., Laub, R.J., and Benson, A.A. (1989) *Lipids 24*, 286–289.
14. Fremont, L., and Marion, D. (1982) *Comp. Biochem. Physiol. 73B*, 849–856.
15. Watanabe, T., Takeuchi, T., Saito, M., and Nishimura, K. (1984) *Bull. Japan. Soc. Sci. Fish. 50*, 1207–1215.
16. Wiegand, M.D., and Idler, D.R. (1985) *Can. J. Zool. 63*, 2775–2777.
17. Wade, M.G., Van Der Kraak, G., Gerrits, M.F., and Ballantyne, J.S. (1994) *Biol. Reprod. 51*, 131–139.
18. Van Der Kraak, G., and Chang, J.P. (1990) *Gen. Comp. Endocrinol. 77*, 221–228.
19. Wade, M.G., and Van Der Kraak, G. (1993) *Gen. Comp. Endocrinol. 90*, 109–118.
20. Meikle, A.W., Benson, S.J., Liu, X.-H., Boam, W.D., and Stringham, J.D. (1989) *Am. J. Physiol. 257*, E937–E942.
21. Johnson, A.L., and Tilly, J.L. (1990) *Biol. Reprod. 42*, 458–464.
22. Johnson, A.L., and Tilly, J.L. (1990) *Biol. Reprod. 43*, 922–928.
23. Speizer, L.A., Watson, M.J., and Brunton, L.L. (1991) *Am. J. Physiol. 261*, E109–E114.
24. Lopez-Ruiz, M.P., Choi, M.S.K., Rose, M.P., West, A.P., and Cooke, B.A. (1992) *Endocrinology 130*, 1122–1130.
25. Elliott, M.E., and Goodfriend, T.L. (1993) *Endocrinology 132*, 2453–2460.
26. Kagawa, H., Young, G., and Nagahama, Y. (1984) *Gen. Comp. Endocrinol. 54*, 139–143.
27. Kime, D.E. (1993) *Rev. Fish Biol. Fish. 3*, 160–180.
28. Van Der Kraak, G. (1992) *J. Exp. Zool. 262*, 271–278.
29. Fontaine, Y.A., Burzawa-Gerard, E., and Delerue-Le Belle, N. (1970) *C.R. Acad. Sci. Paris Ser. D271*, 780–783.
30. Kanamori, A., and Nagahama, Y. (1988) *Gen. Comp. Endocrinol. 72*, 39–53.
31. Srivastava, R.K., and Van Der Kraak, G. (1994) *Gen. Comp. Endocrinol. 95*, 60–70.
32. Rennert, H., Amsterdam, A., Billheimer, J.T., and Strauss III, J.F. (1991) *Biochemistry 30*, 11280–11285.
33. Yamamoto, R., Kallen, C.B., Babalola, G.O., Rennert, H., Billheimer, J.T., and Strauss III, J.F. (1991) *Proc. Natl. Acad. Sci. USA 88*, 463–467.
34. Sordoillet, C., Chauvin, M.A., Hendrick, J.C., Franchimont, P., Morera, A.M., and Benahmed, M. (1992) *Endocrinology 130*, 1352–1358.
35. Engelhardt, H., Tekpetey, F.R., Gore-Langton, R.E., and Armstrong, D.T. (1992) *Mol. Cell. Endocrinol. 85*, 117–126.
36. Mauduit, C., Chauvin, M.A., Hartmann, D.J., Revol, A., Morera, A.M., and Benahmed, M. (1992) *Biol. Reprod. 46*, 1119–1126.
37. Golos, T.G., Strauss III, J.F., and Miller, W.L. (1987) *J. Steroid Biochem. 27*, 767–773.
38. Nestler, J.E., Takagi, K., and Strauss III, J.F. (1990) in *Advances in Cholesterol Research* (Esfahani, M., and Swaney, J.B., eds.) pp. 133–170, The Caldwell Press, New Jersey.
39. Billheimer, J.T., and Gillies, P.J. (1990) in *Advances in Cholesterol Research* (Esfahani, M., and Swaney, J.B., eds.) pp. 7–46, The Caldwell Press, New Jersey.
40. Leung, P.C.K., and Steele, G.L. (1992) *Endo. Rev. 13*, 476–498.
41. Steele, G.L., and Leung, P.C.K. (1993) in *The Ovary* (Adashi, E.Y., and Leung, P.C.K., eds.) pp. 113–127, Raven Press, Ltd., New York.
42. Ranjan, M., and Goetz, F.W. (1987) *J. Exp. Zool. 242*, 355–361.
43. Van Der Kraak, G. (1990) *Biol. Reprod. 42*, 231–238.
44. Van Der Kraak, G. (1991) *Gen. Comp. Endocrinol. 81*, 268–275.
45. Srivastava, R.K., and Van Der Kraak, G. (1994) *Gen. Comp. Endocrinol. 93*, 181–191.
46. Goetz, F.W. (1993) *Biol. Reprod. 48*, 846–850.
47. Nishizuka, Y. (1984) *Nature 308*, 693–697.
48. Berridge, M.J. (1984) *Biochem. J. 220*, 345–360.
49. Wade, M.G., and Van Der Kraak, G. (1995) *Biol. Reprod. 51*, 131–139.
50. Greene, D.H., and Selivonchick, D.P. (1987) *Prog. Lipid Res. 26*, 53–85.

51. Orton, T.C., and Parker, G.L. (1982) *Drug Metab. Dispos. 10*, 110–115.
52. Reddy, J.K., Goel, S.K., Nemali, M.R., Carrino, J.J., Laffler, T.G., Reddy, M.K., Sperbeck, S.J., Osumi, T., Hashimoto, T., Lalwni, N.D., and Rao, M.S. (1986) *Proc. Natl. Acad. Sci. USA 83*, 1747–1751.
53. Issemann, I., and Green, S. (1990) *Nature 347*, 645–650.
54. Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J.-A. (1992) *Proc. Natl. Acad. Sci. USA 89*, 4653–4657.
55. Issemann, I., Prince, R.A., Tugwood, J.D., and Green, S. (1993) *J. Mol. Endocrinol. 11*, 37–47.
56. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahli, W. (1993) *Proc. Natl. Acad. Sci. USA 90*, 2160–2164.
57. Lock, E.A., Mitchell, A.M., and Elcombe, C.R. (1989) *Annu. Rev. Pharmacol. Toxicol. 29*, 145–163.
58. Auwerx, J. (1992) *Horm. Res. 38*, 269–277.
59. Bell, D.R., and Elcombe, C.R. (1993) in *Peroxisomes: Biology and Importance in Toxicology and Medicine* (Gibson, G., and Lake, B., eds.) pp. 137–148, Taylor and Francis, Washington, D.C.
60. Ballantyne, J.S., Glemet, H.C., Chamberlin, M.E., and Singer, T.D. (1993) *Mar. Biol. 116*, 47–52.

[Received September 7, 1994, and in revised form February 27, 1995; Revision accepted March 22, 1995]

Occurrence of 22:3n-9 and 22:4n-9 in the Lipids of the Topminnow *(Poeciliopsis lucida)* Hepatic Tumor Cell Line, PLHC-1

Douglas R. Tocher*, James R. Dick and John R. Sargent

NERC Unit of Aquatic Biochemistry, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland

ABSTRACT: The lipids of the hepatic tumor cell line, PLHC-1, from the topminnow *(Poeciliopsis lucida),* were found to contain considerable amounts of a range of n-9 polyunsaturated fatty acids despite culture in serum containing significant amounts of essential fatty acids. The structural identity of all the n-9 polyunsaturated fatty acids was confirmed by gas chromatography/mass spectrometry. Of particular interest, PLHC-1 cell total lipid contained 1.9% of 22:3n-9 and 3.3% of 22:4n-9. As the culture medium contained virtually no n-9 polyunsaturated fatty acids, these fatty acids are all formed by the PLHC-1 cells, presumably from 18:1n-9. The 22:3n-9 and 22:4n-9 are presumably formed by processes of elongation and "Δ4" desaturation of Mead acid, 20:3n-9, present at over 11% in fatty acids of total lipid. Both 22:3n-9 and 22:4n-9 were primarily located in phosphatidylserine (4.1 and 8.5%, respectively) and, to a lesser extent, in phosphatidylethanolamine (2.2 and 6.5%, respectively), in common with the C_{22} derivatives of the n-3 and n-6 series, whereas 20:3n-9 was preferentially located in phosphatidylinositol (31.2%). The results establish that long-chain polyunsaturated fatty acids of the n-9 series can be formed in vertebrate tissue other than in conditions of classical essential fatty acid deficiency.

Lipids 30, 555–565 (1995).

Animals, including fish, lack the Δ12 and Δ15 fatty acyl desaturase enzymes required for the production of 18:2n-6 and 18:3n-3 from 18:1n-9 and, hence, have an essential dietary requirement for these polyunsaturated fatty acids (PUFA) (1,2). In general, animals have the ability to desaturate and elongate the C_{18} essential fatty acids (EFA) to longer-chain, more unsaturated derivatives such as 20:4n-6, generally the major PUFA in terrestrial mammals (3), or 20:5n-3 and 22:6n-3, generally the major PUFA in fish (4–6). In EFA deficiency, animals synthesize n-9 PUFA derived from 18:1n-9 which, under normal conditions, is a process suppressed by the presence of n-3 and n-6 PUFA (7–9). The normal end-product of the metabolism of 18:1n-9 *via* the desaturase/elongase pathways is Mead acid (10), 20:3n-9, which accumulates in mammals in the place of 20:4n-6 (11). Mead acid also accumulates in salmonids and other freshwater fish placed on EFA-deficient diets (12,13). There is no evidence for any significant metabolism of Mead acid to longer-chain, more unsaturated fatty acids in EFA-deficient animals (11,14).

Cell culture media are normally devoid of any fatty acids so that cells in culture generally derive all their lipid and fatty acids from the lipid contained in the serum supplement. Sera are lipid-rich, and fetal calf serum (FCS), the predominant serum supplement in use, is relatively rich in PUFA (15,16). For mammalian cells, FCS provides a sufficient amount and balance of n-6 and n-3 PUFA. In contrast, although the total amount of PUFA is adequate, fish cells grown in FCS display lower percentages of n-3 PUFA and are enriched in n-6 PUFA in comparison with fish tissues (15). Most fish cell lines grown in FCS contain only small amounts of n-9 PUFA, generally 20:3n-9 (15).

In the present paper, we report on a fish cell line, PLHC-1 (17), derived from hepatocarcinoma tissue from the topminnow (*Poeciliopsis lucida*), which, under standard, routine culture conditions in medium containing 10% FCS, contained considerable amounts of n-9 PUFA and, in particular, significant amounts of 22:3n-9 and 22:4n-9.

MATERIALS AND METHODS

Cell lines and media. The cell line, PLHC-1, was initially cultured in 1985 *via* trypsinization of a differentiated hepatocellular tumor from the topminnow (*P. lucida*) (17). The cells are epithelial but retain some of the characteristic morphology of primary liver hepatocytes. The cells used in the present study had been subcultured for 90–100 passages. The PLHC-1 cells were maintained in CO_2-Independent Medium (a proprietary product obtained from Gibco BRL, Life Technologies Ltd., Paisley, Scotland) supplemented with 2 mM glutamine, antibiotics (50 I.U. mL^{-1} penicillin and 50 mg · mL^{-1} streptomycin), and 10% FCS. The cells used in

*To whom correspondence should be addressed at NERC Unit of Aquatic Biochemistry, Dept. of Biological and Molecular Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom.

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CI, chemical ionization; EFA, essential fatty acids; EI, electron ionization; FAME, fatty acid methyl esters; FCS, fetal calf serum; GC, gas chromatography; HBSS, Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}); MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TF, turbot fin cell line.

Copyright © 1995 by AOCS Press

the present study were a gift from Dr. L.E. Hightower (Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT).

Incubation conditions. Cells were cultured at 30°C in sealed plastic tissue culture flasks (Greiner Laboritechnik Ltd., Dursley, United Kingdom). For routine cultivation of the cells for provision of experimental material, cells were cultured in 75-cm^2 flasks and were subcultured within 24 h of reaching confluence. For lipid analyses, cells were subcultured into 175-cm^2 flasks at a seeding density of 1×10^5 cells · cm^{-2}. For each sample for full lipid analyses, 4×175 cm^2 flasks were seeded, and this experiment was repeated three times to obtain the replicates. Therefore, all results are means ± SD of three such experiments.

Lipid extraction. The medium was aspirated, and the cultures were washed twice with 20 mL of ice-cold Hanks' balanced salt solution (HBSS) without calcium and magnesium. Cells were harvested by trypsinization with 0.05% trypsin/0.5 mM EDTA, washed with 5 mL ice-cold HBSS containing 1% fatty acid-free bovine serum albumin (BSA) and counted as described in Tocher *et al.* (18). Total lipid was determined gravimetrically after extraction with chloroform/methanol (2:1, vol/vol) essentially by the method of Folch *et al.* (19) as described in detail previously (15). All solvents contained 0.01% butylated hydroxytoluene (BHT) as antioxidant.

Lipid class analysis. Lipid classes were separated by one-dimensional, double-development high-performance thin-layer chromatography using methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) and hexane/diethyl ether/acetic acid (85:15:1.5, by vol) as developing solvents as described previously (20). Lipid classes were quantified by charring (21), followed by calibrated scanning densitometry using a Shimadzu CS-9000 dual wavelength flying spot scanner and DR-2 recorder (Tokyo, Japan) (22).

Separation of glycerophospholipid and neutral lipid classes. Samples of total lipid (3–4 mg) were applied in 4-cm streaks to a thin-layer chromatography plate, and the polar lipid classes were separated using methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) as developing solvent (23). Lipid classes were visualized under ultraviolet after spraying with 2,7-dichlorofluorescein. Individual glycerophospholipid classes were scraped into stoppered test tubes and transmethylated directly on the silica after addition of 1 mL toluene and 2.5 mL 1% sulfuric acid in methanol, and fatty acid methyl esters (FAME) prepared as described below. Total neutral lipid classes which run at the solvent front in the above solvent system were scraped and eluted from the silica using chloroform/methanol (2:1, vol/vol) before transmethylation, as described below.

Fatty acid analyses. FAME were prepared from total lipid and individual lipid classes by acid-catalyzed transmethylation at 50°C for 16 h (see Ref. 24) and were extracted and purified as described previously (20). Methyl esters were analyzed in a Carlo Erba Vega GC6000 gas chromatograph (Carlo Erba Instrumentazone, Milan, Italy) equipped with a CP Wax 51 fused silica capillary column (50 m × 0.32 mm i.d.; Chrompack U.K. Ltd., London, England), with on-column injection using hydrogen as carrier gas and a biphasic thermal gradient from 50 to 225°C. Routinely, FAME were identified by comparison with known standards and a well-characterized fish oil and by reference to published data as described previously (20), and were quantified using a Carlo Erba DP800 data processor. All solvents contained 0.01% BHT as an antioxidant.

Gas chromatography/mass spectrometry (GC/MS). The identities of fatty acids were confirmed by subjecting the methyl or picolinyl esters (25) to GC/MS using a Carlo Erba 8000 gas chromatograph coupled to an MD800 mass spectrometer (Fisons Instruments, Crawley, United Kingdom) (see Ref. 26). Methyl esters were analyzed using chemical ionization (CI) using methane as reagent gas, and picolinyl esters were analyzed using the electron ionization (EI) mode at 70 eV. The gas chromatograph was equipped with a fused silica capillary column (15 m × 0.25 mm i.d.) coated with DB 5MS (J&W Scientific, Folsom, CA) and used helium as carrier gas. Samples were applied using on-column injection with the oven temperature programmed to rise from 50 to 260°C.

Materials. HBSS, glutamine, antibiotics, FCS, trypan blue, trypsin/EDTA, fatty acid-free BSA, and BHT were obtained from Sigma Chemical Co. Ltd. (Dorset, United Kingdom). Thin-layer chromatography (20- × 20-cm × 0.25-mm) and high-performance thin-layer chromatography (10- × 10-cm × 0.15-mm) plates precoated with silica gel 60 (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany). All solvents were high-performance liquid chromatography-grade and were from Rathburn Chemicals (Peebleshire, United Kingdom).

RESULTS

Lipid content and lipid class composition. PLHC-1 cells contained almost 21 μg of total lipid per million cells (Table 1). Polar lipid classes predominated, accounting for 81.5% of the total lipid, with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) together amounting to almost 54% of the total lipid, with approximately 8% each of phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (Table 1). Cholesterol was the major neutral lipid at about 13% with the storage lipids, triacylglycerol and steryl ester, accounting for less than 5 and 1% of the total lipid, respectively (Table 1).

Fatty acid composition of total lipid. The fatty acid composition of the total lipid was characterized by approximately equal proportions of saturated, monounsaturated, and PUFA (Table 2). The total for saturated fatty acids was mainly due to 16:0 and 18:0 and the monounsaturated fatty acids were predominantly 18:1n-9 (Table 2). Total n-3 and n-6 PUFA were both present at about 5% of the total fatty acids with 22:6n-3 (3.8%) and 20:4n-6 (3.0%) being the main components, respectively (Table 2). However, the predominant

TABLE 1
Lipid Content and Lipid Class Composition (percentage of total lipid) of Hepatic Tumor Cell Line, PLHC-1, Cells Grown at 30°C in Medium Supplemented with 10% Fetal Calf Serum[a]

Lipid class	Content
Total lipid content (μg/10^6 cells)	20.7 ± 1.7
Phosphatidylcholine	28.3 ± 0.6
Phosphatidylethanolamine	25.5 ± 1.6
Phosphatidylserine	7.5 ± 0.1
Phosphatidylinositol	8.7 ± 0.1
Phosphatidic acid/cardiolipin	2.7 ± 0.5
Sphingomyelin	7.6 ± 0.3
Sulfatides/cerebrosides	1.2 ± 0.2
Total polar lipids	81.5 ± 0.1
Total neutral lipids	18.5 ± 0.1
Cholesterol	12.9 ± 0.4
Triacylglycerol	4.7 ± 1.2
Steryl ester	0.9 ± 0.7
Free fatty acids	n.d.

[a]Results are means ± SD (n = 3); n.d., none detected.

TABLE 2
Fatty Acid Compositions (percentage of weight) of Fetal Calf Serum and Total Lipid from PLHC-1 Cells Grown at 30°C in Medium Supplemented with 10% Fetal Calf Serum[a]

Fatty acid	Fetal calf serum	PLHC-1
14:0	1.3 ± 0.0	0.8 ± 0.0
15:0	0.9 ± 0.0	0.5 ± 0.1
16:0	23.7 ± 0.4	12.5 ± 0.6
17:0	1.1 ± 0.1	0.6 ± 0.1
18:0	15.2 ± 0.2	13.9 ± 0.3
20:0	0.4 ± 0.1	n.d.
24:0	0.6 ± 0.1	n.d.
Total saturated	43.2 ± 0.4	28.3 ± 0.5
16:1n-9	1.7 ± 0.1	0.2 ± 0.0
16:1n-7	2.1 ± 0.1	7.7 ± 0.1
18:1n-9	17.0 ± 0.9	20.5 ± 0.1
18:1n-7	5.0 ± 0.1	2.7 ± 0.0
20:1n-9	0.4 ± 0.0	1.0 ± 0.0
20:1n-7	0.2 ± 0.0	0.2 ± 0.0
22:1	1.1 ± 0.0	0.1 ± 0.1
24:1	2.8 ± 0.4	0.7 ± 0.1
Total monounsaturated	30.3 ± 1.4	33.1 ± 0.4
18:2n-9	n.d.	3.7 ± 0.2
20:2n-9	0.6 ± 0.1	1.5 ± 0.1
20:3n-9	n.d.	11.1 ± 0.1
22:2n-9	n.d.	n.d.
22:3n-9	n.d.	1.9 ± 0.1
22:4n-9	n.d.	3.3 ± 0.1
Total n-9 PUFA	0.6 ± 0.1	21.5 ± 0.2
18:2n-6	2.8 ± 0.0	0.3 ± 0.1
20:2n-6	0.6 ± 0.0	0.3 ± 0.0
20:3n-6	2.1 ± 0.0	0.2 ± 0.0
20:4n-6	6.1 ± 1.3	3.0 ± 0.4
22:4n-6	0.5 ± 0.1	0.4 ± 0.0
22:5n-6	1.3 ± 0.6	0.9 ± 0.1
Total n-6 PUFA	13.4 ± 0.6	5.1 ± 0.2
18:3n-3	0.5 ± 0.1	n.d.
18:4n-3	0.2 ± 0.0	n.d.
20:4n-3	0.3 ± 0.1	0.1 ± 0.1
20:5n-3	1.1 ± 0.1	0.4 ± 0.0
22:5n-3	3.7 ± 0.1	0.7 ± 0.0
22:6n-3	5.2 ± 1.0	3.8 ± 0.4
Total n-3 PUFA	11.0 ± 0.8	5.0 ± 0.5
Total dimethylacetals	n.d.	3.6 ± 0.1
Total PUFA	25.0 ± 1.5	31.6 ± 0.8
Total unidentified	1.5 ± 0.4	3.4 ± 0.9

[a]Results are means ± SD (n = 3). PUFA, polyunsaturated fatty acids; n.d., none detected.

PUFA in PLHC-1 cells was 20:3n-9, which accounted for over 11% of total fatty acids with total n-9 PUFA amounting to 21.5% of the total fatty acids (Table 2). PLHC-1 cells also contained 3.7% 18:2n-9, but of most interest was the presence of 1.9% 22:3n-9 and 3.3% 22:4n-9 (Table 2). Culture of the cells in lipid-free medium increased the percentage of total n-9 PUFA to 25.1 ± 0.7%, with 20:2n-9 and 22:3n-9 increased 3-fold and 1.5-fold, respectively, all increases being significant (data not shown). The percentages of 18:2n-9 and 20:3n-9 were slightly, but not significantly, increased, but the percentage of 22:4n-9 decreased in lipid-free culture.

Identification of 22:4n-9 by GC/MS. Figure 1 shows the analytical gas chromatogram of PLHC-1 total lipid FAME with the positions of the major and unusual (putative n-9 PUFA) fatty acids marked. The n-9 PUFA were all subjected to GC/MS in order to positively identify their structure. The GC trace from the GC/MS for PLHC-1 cell total lipid FAME shows C_{22} PUFA as a cluster of peaks between positions 3259 and 3356 (Fig. 2A). CI+ was used to generate molecular (base) ions to determine molecular weights. Peak 3283 gave a molecular (base) ion of molecular weight 347 corresponding to 22:4 (Fig. 2B). CI also established (data not shown) that peaks 3259 and 3356 in Figure 2A were 22:6 and 22:3, respectively. The shoulder on the front of 3259 was 22:5, and the two peaks between 3283 and 3356 were, in elution order, further 22:4 and 22:5 fatty acids.

To identify the double-bond positions of the various C_{22} PUFA, picolinyl esters were prepared and subjected to GC/MS (Fig. 3A) (25). Peak 3751 in the picolinyl trace corresponded to peak 3283 in the FAME trace. EI analysis of peak 3751 showed a mass ion of 423 *m/z*, and fragments at 394, 380, 366, 352, 338, 324, 310, 296, and then 284 *m/z* (Fig. 3B). This pattern with an initial interval of 29 for the first main fragment (15 for the terminal methyl plus 14 for the first methylene) followed by intervals of 14 (methylenes) is characteristic of the fragmentation of picolinyl esters of fatty acids (see Refs. 25,26). The interval of 26 (14 + 12) between 310 and 284 denotes the position of the first double bond, indicating that this fatty acid is n-9. Therefore peak 3751was confirmed as 22:4n-9. The EI spectra of peaks 2785 and 3894 are shown in Figure 4A and B, respectively. Peak 2785 showed a fragmentation pattern with a mass ion of 397 *m/z* and peaks at 368, 354, 340, 326, 312, 298, 284, 270, and 258 (Fig. 4A). Peak 2785 was known to be 20:3n-9, and this pat-

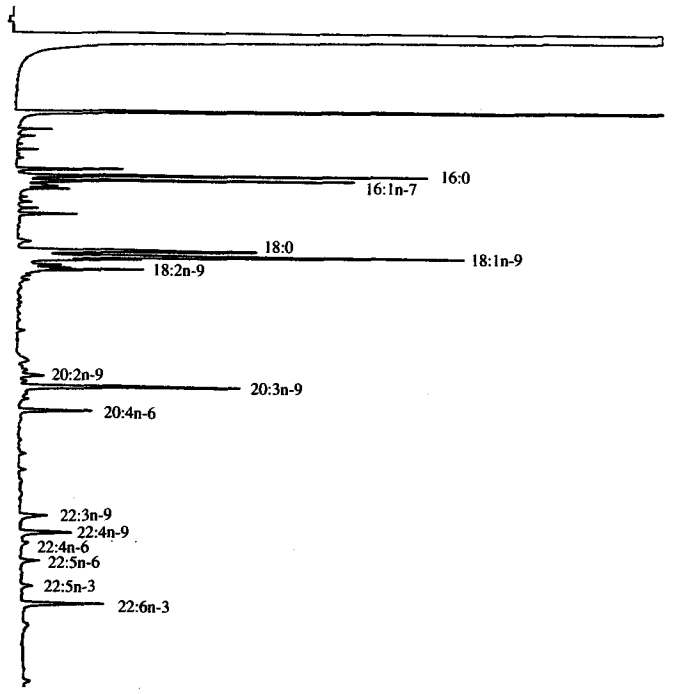


FIG. 1. Capillary gas chromatograph of fatty acid methyl esters (FAME) prepared from total lipid from PLHC-1 cells cultured at 30°C in medium containing 10% fetal calf serum. Lipid was extracted, FAME prepared, and gas chromatography performed as described in the Materials and Methods section.

tern with the 26 (14 +12) interval between 284 and 258 confirmed its structure as n-9. Peak 3894 was known from the CI analysis to be 22:3, and the EI fragmentation pattern of the picolinyl ester with mass ion of 425 *m/z* and fragments at 396, 382, 368, 354, 340, 326, 312, 298, and then 286 *m/z* confirmed its structure as n-9 (Fig. 4B).

Fatty acid compositions of glycerophospholipids and neutral lipid in PLHC-1 cells. The fatty acid compositions of the major phosphoglycerides showed that all the C_{22} PUFA were found in the greatest percentages in PS with 22:3n-9 and 22:4n-9 accounting for 4.1 and 8.5% of total fatty acids in PS, respectively (Table 3). However, the highest percentage (35.4%) of total n-9 PUFA was found in PI due to a highly selective localization of 20:3n-9 which made up over 31% of total PI fatty acids (Table 3). The highest percentage of arachidonic acid (20:4n-6) was also found in PI. The major non-PUFA in PS and PI was 18:0, accounting for some 39 and 34% of their total fatty acids, respectively.

Total neutral lipids had the lowest levels of PUFA, with over 81% of total fatty acids being saturated or monounsaturated, predominantly 18:1n-9, 16:0, and 16:1(n-7) (Table 3). A very similar composition to that of total neutral lipids was found in PC which was also characterized by having the highest percentage (5.7%) of 18:2n-9 (Table 3). PE had the lowest percentage of saturated fatty acids, intermediate levels of monounsaturated fatty acids, n-9 and n-3 PUFA, but had the highest percentage of total n-6 PUFA due to the specific localization of 20:3n-6 (5.5%) (Table 3).

DISCUSSION

The present paper reports on the presence of 22:3n-9 and 22:4n-9 in a cultured hepatoma cell line from a fish, the topminnow (*P. lucida*). The unequivocal characterization of these two unusual PUFA was made by GC/MS of picolinyl esters of the fatty acids. The resolution of picolinyl esters by the GC was not as high as with FAME, and this resulted in very close elution of the picolinyl esters of 22:4n-9 and 22:6n-3. However, culture of the PLHC-1 cells in lipid-free medium resulted in the almost complete loss from the cells of the n-3 and n-6 C_{22} PUFA including 22:6n-3 (data not shown). This resulted in a much simpler GC trace in the region of the C_{22} PUFA so that the 22:4n-9 peak (peak 3751 in Fig. 3A) was >99% 22:4n-9. Therefore, culture in lipid-free conditions facilitated positive identification of the n-9 PUFA by GC/MS. However, the data shown here establish positive identification of the n-9 PUFA in PLHC-1 cells under routine culture conditions, i.e., in medium containing serum. Culture of the cells in lipid-free medium also suggested that oleate in the medium was not the primary source of the n-9 PUFA in the PLHC-1 cells as total n-9 PUFA were increased in lipid-free culture.

Under normal culture conditions, in the presence of 10% FCS, the 22:3n-9 and 22:4n-9 were present at a combined level of over 5% of the total fatty acids. Neither of these PUFA is present in FCS. Indeed PUFA of the n-9 series are virtually absent in FCS, with only 0.6% of 20:2n-9 found in the batch of FCS used in these experiments. Thus, the 22:3n-9 and 22:4n-9 are the result of endogenous metabolism of 18:1n-9 in the PLHC-1 cells. It is probable that they arise *via* elongation of 20:3n-9 followed by a "Δ4 desaturation" in a situation paralleling the formation of 22:4n-6/22:5n-6 and 22:5n-3/22:6n-3. Studies have shown that 20:3n-9 can be a substrate for the fatty acid chain elongation system of mammalian liver endoplasmic reticulum and at a rate similar to that for the elongation of 20:4n-6 (27). Banerjee and Rosenthal (28) found that, in human skin fibroblasts in culture, the rate of elongation of 20:3n-9 to 22:3n-9 was intermediate between the rates of elongation of 20:5n-3 (which was faster) and 20:4n-6 (slower). This is different to the situation with most desaturases where the rank order for relative rates of activity is n-3 > n-6 > n-9 (14,29). However, there is nothing known about the desaturation of 22:3n-9 to 22:4n-9. Recently, Sprecher and co-workers (30–32) have characterized a pathway for the desaturation of 22:5n-3 to 22:6n-3 in rat liver independent of a Δ4 desaturase activity. This pathway involves microsomal elongation of 22:5n-3 to 24:5n-3 followed by microsomal desaturation to 24:6n-3 *via* a Δ6 desaturase activity and finally partial β-oxidation, probably peroxisomal, to yield 22:6n-3 (30). In the present study with the columns used in the GC/MS, it would have been possible to detect the presence of very small amounts of C_{24} PUFA. However, if any C_{24} PUFA was present in the PLHC-1 cells, it was below detection limit. Therefore, the pathway for the formation of 22:4n-9 in PLHC-1 cells is not known.

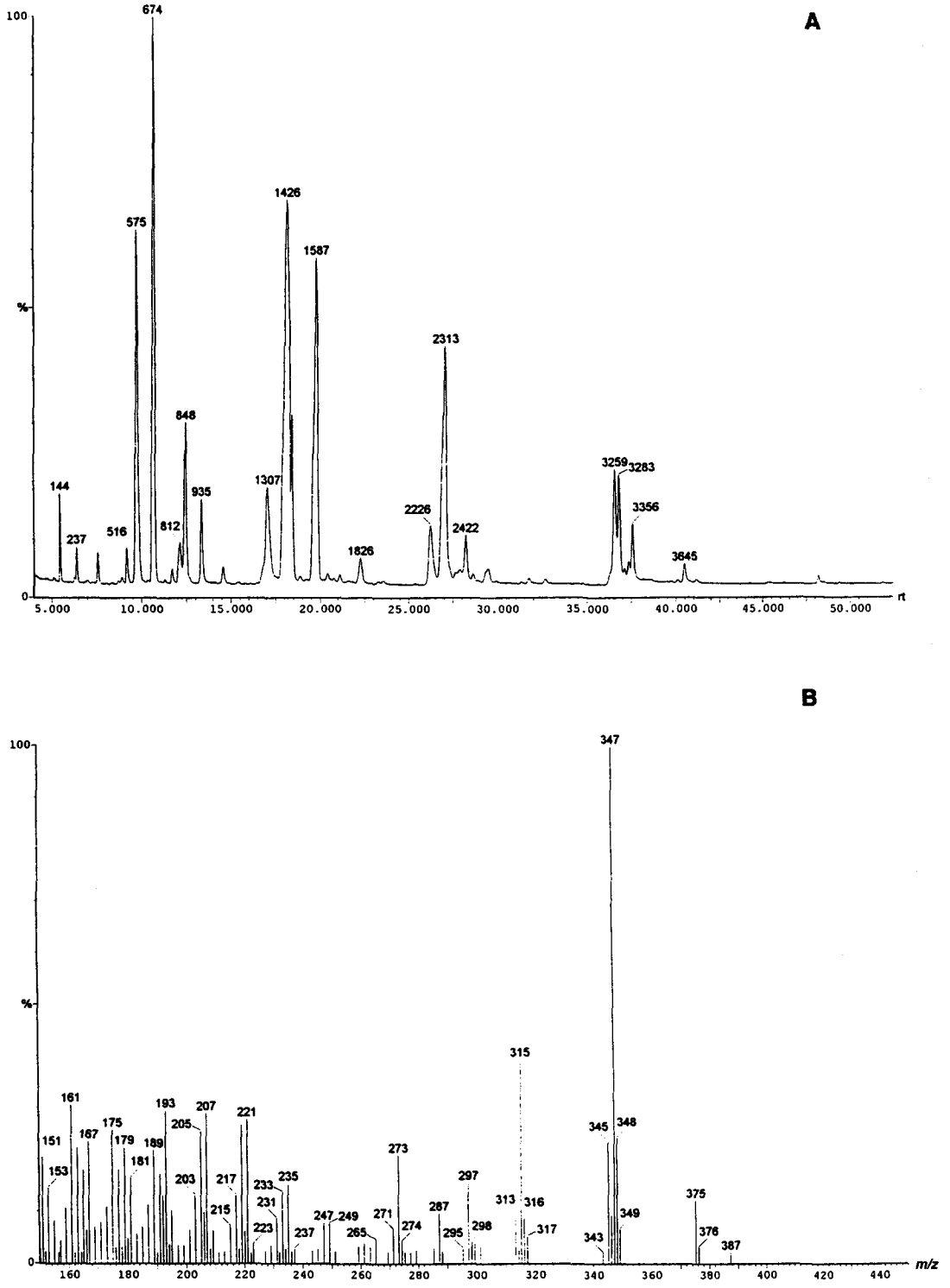


FIG. 2. Capillary gas chromatography of total lipid fatty acid methyl esters from PLHC-1 cells (A) and the chemical ionization mass spectrum of 22:4n-9 (peak 3283) (B). Cell culture conditions and gas chromatography/mass spectrometry procedures as described in the Materials and Methods section; rt, retention time.

Neither of these very long-chain metabolites of 20:3n-9 has been commonly found in animal tissues where Mead acid has long been regarded as the end product of the metabolism of 18:1n-9 *via* the classical elongation/desaturation pathways (7,8). Furthermore, the metabolism of 18:1n-9 by the desaturase/elongase pathway and the production of Mead acid generally only occurs in conditions of EFA deficiency (7,8,11). For instance, in rats fed for 5 mon on an EFA-deficient diet (hydrogenated coconut oil), 20:3n-9 accumulated in erythrocytes in the place of 20:4n-6, and only very small amounts of two minor fatty acids that behaved chromatographically similar to 22:3n-9 and 22:4n-9 were found (11). An EFA-defi-

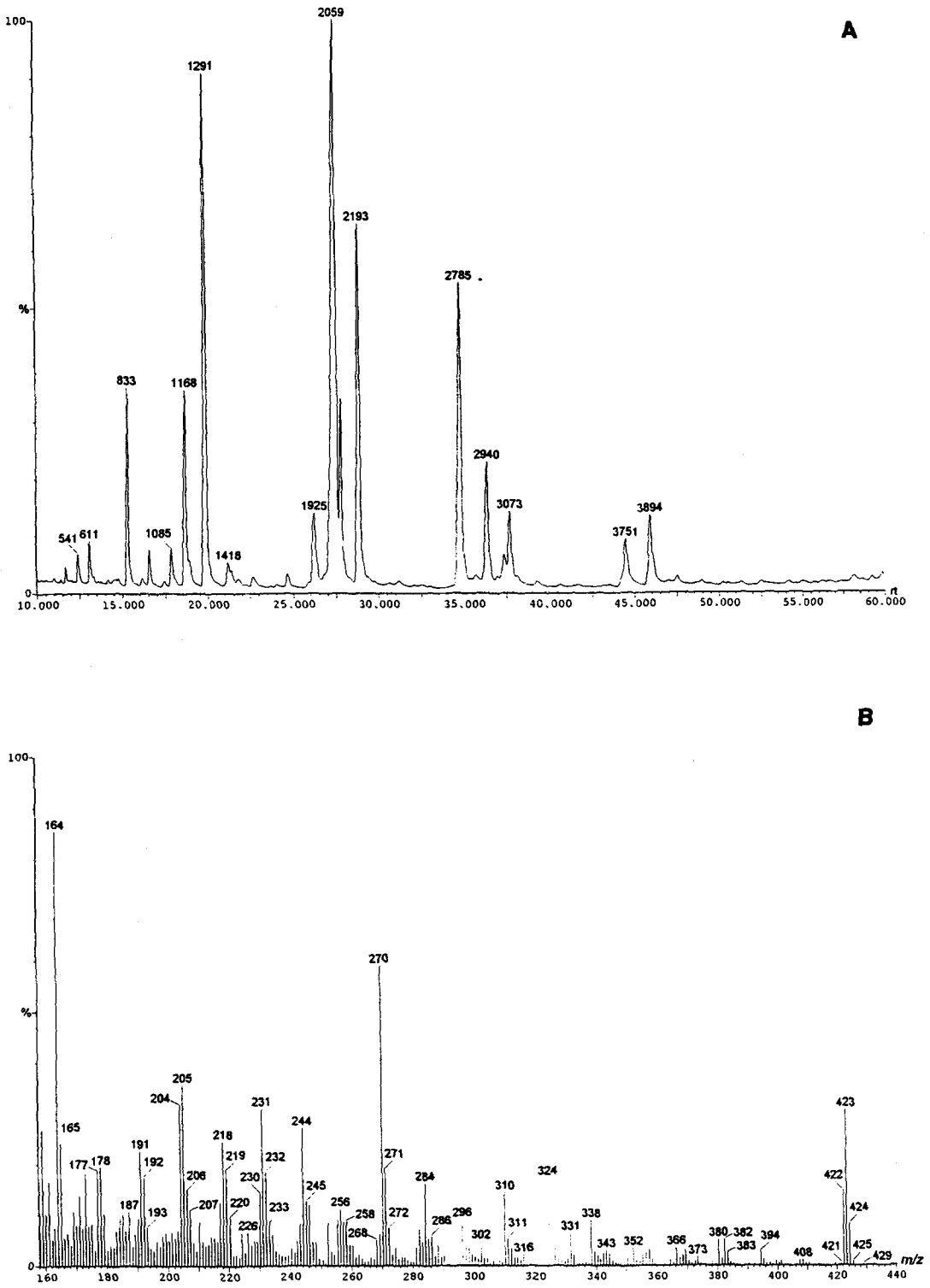


FIG. 3. Capillary gas chromatography of picolinyl esters of total lipid fatty acids from PLHC-1 cells (A) and electron ionization (70 eV) mass spectrum of 22:4n-9 (peak 3751) (B). Cell culture conditions and gas chromatography/mass spectrometry procedures as described in the Materials and Methods section; rt, retention time.

ciency-like profile of fatty acids, with increased 20:3n-9, has been observed in human skin fibroblasts when cultured at low density in culture medium containing low levels (0.4%) of FCS (33–35). No 22:4n-9 was detected in the human skin fibroblasts, but the cells were interesting in that they also produced measurable 22:3n-9 as well as 20:3n-9 under these low-lipid conditions (33–35).

However, although they contain substantial amounts of 20:3n-9, it is not likely that the PLHC-1 cells are in a state of EFA-deficiency. FCS delivers substantial amounts of EFA

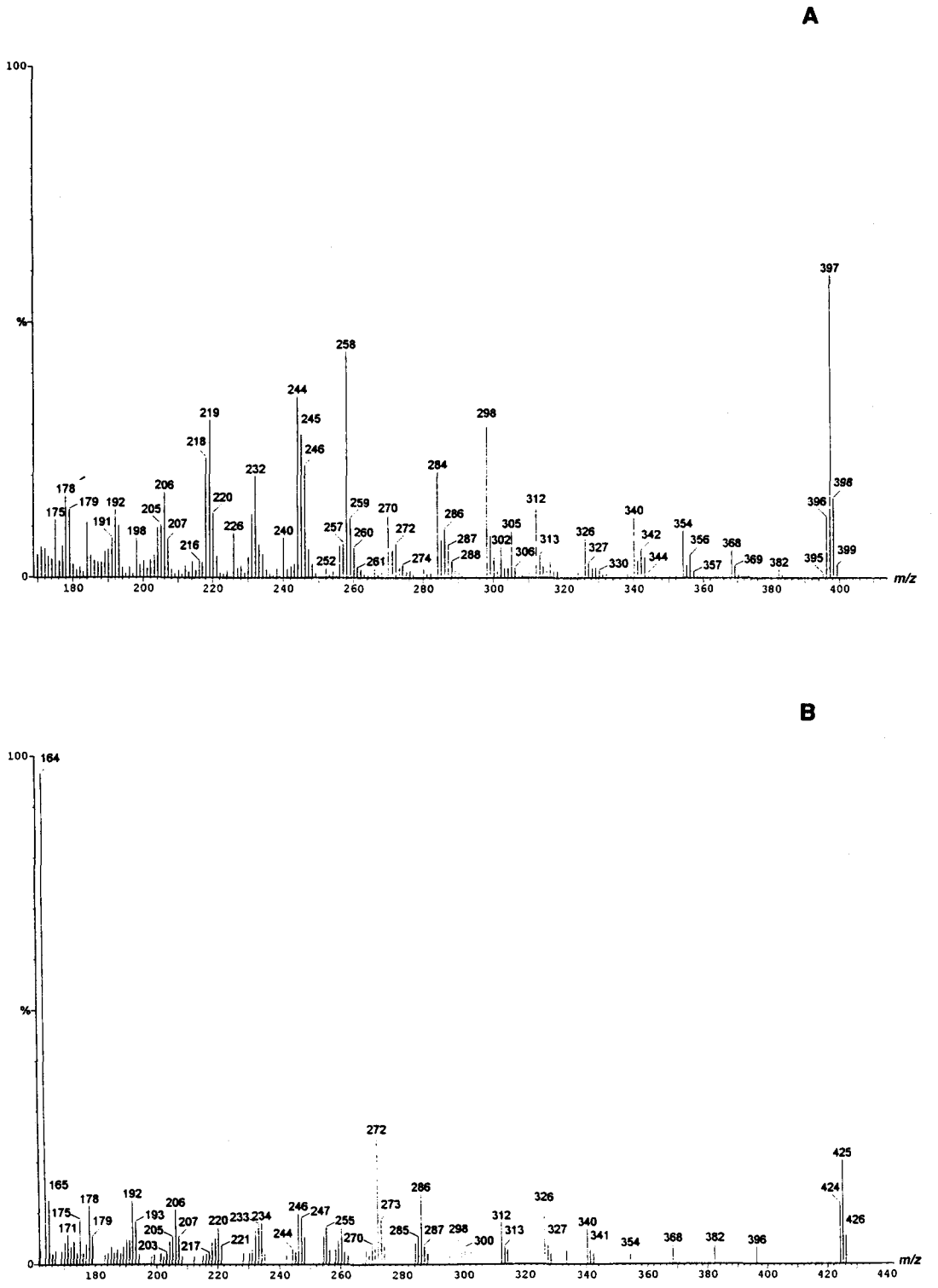


FIG. 4. Electron ionization (70 eV) mass spectra of 20:3n-9 (peak 2785 in Fig. 3A) (A) and 22:3n-9 (peak 3894 in Fig. 3A) (B). Cell culture conditions and gas chromatography/mass spectrometry procedures as described in the Materials and Methods section.

(2.8% 18:2) and, especially, significant amounts of C_{20} and C_{22} PUFA of the n-3 (3.7% 22:5 and 5.2% 22:6) and n-6 series (6.1% 20:4). In quantitative terms, the batch of FCS used contained approximately 4 mg of total lipid per mL of serum. Based on the lipid and fatty acid composition of the serum, approximately 50% (2 mg) of the serum was total fatty acid, a quarter of which was total PUFA. Therefore, the serum contained about 500 μg of PUFA/mL, and so the medium contained 50 μg of PUFA/mL. At confluence, PLHC-1 cells were present at 0.5×10^6 cells/cm^2 (representing about 5 μg of total

TABLE 3
Fatty Acid Compositions (percentage of weight) of the Major Glycerophospholipids and Total Neutral Lipids from PLHC-1 Cells Grown at 30°C in Medium Supplemented with 10% Fetal Calf Serum[a]

Fatty acid	Lipid class				
	PC	PE	PS	PI	TN
14:0	1.3 ± 0.0	0.3 ± 0.1	n.d.	0.1 ± 0.0	2.0 ± 0.1
15:0	0.8 ± 0.0	1.1 ± 0.4	0.1 ± 0.1	0.1 ± 0.0	1.2 ± 0.1
16:0	22.4 ± 0.9	5.5 ± 0.3	4.4 ± 0.2	4.0 ± 0.1	21.6 ± 0.3
17:0	0.6 ± 0.1	0.8 ± 0.1	0.5 ± 0.4	0.4 ± 0.4	1.1 ± 0.2
18:0	9.5 ± 1.1	17.4 ± 0.3	39.3 ± 0.5	34.1 ± 2.0	8.9 ± 0.3
Total saturated	34.6 ± 0.2	25.1 ± 1.1	44.3 ± 1.0	38.7 ± 1.3	34.8 ± 0.0
16:1[b]	14.9 ± 0.1	4.5 ± 0.8	1.4 ± 0.7	2.5 ± 0.2	11.7 ± 0.2
18:1n-9	27.7 ± 1.6	17.8 ± 1.7	10.1 ± 1.2	9.4 ± 0.7	24.0 ± 0.3
18:1n-7	2.9 ± 0.6	3.7 ± 0.6	1.1 ± 0.4	2.1 ± 0.6	3.6 ± 0.0
20:1n-9	0.7 ± 0.3	1.2 ± 0.1	0.3 ± 0.3	1.3 ± 0.9	2.7 ± 0.2
20:1n-7	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	n.d.	0.1 ± 0.1
22:1	0.1 ± 0.1	n.d.	n.d.	n.d.	1.9 ± 0.1
24:1	0.6 ± 0.3	n.d.	n.d.	n.d.	2.3 ± 0.3
Total monounsaturated	47.0 ± 1.6	27.4 ± 1.5	13.0 ± 0.6	15.3 ± 2.5	46.3 ± 0.6
18:2n-9	5.7 ± 0.4	3.4 ± 0.6	3.0 ± 0.2	0.6 ± 0.1	3.1 ± 0.2
20:2n-9	0.5 ± 0.1	1.7 ± 0.2	1.8 ± 0.0	0.5 ± 0.2	1.9 ± 0.1
20:3n-9	6.3 ± 0.0	12.9 ± 0.0	2.8 ± 0.2	31.2 ± 1.8	3.4 ± 0.2
22:3n-9	0.6 ± 0.0	2.2 ± 0.2	4.1 ± 0.5	2.3 ± 0.0	1.7 ± 0.1
22:4n-9	0.7 ± 0.0	6.5 ± 0.3	8.5 ± 0.9	0.8 ± 0.1	1.0 ± 0.0
Total n-9 PUFA	13.8 ± 0.3	26.7 ± 0.6	20.2 ± 1.4	35.4 ± 0.7	11.1 ± 0.6
18:2n-6	0.1 ± 0.1	n.d.	n.d.	n.d.	0.5 ± 0.1
20:2n-6	0.2 ± 0.2	n.d.	n.d.	n.d.	n.d.
20:3n-6	0.1 ± 0.1	5.5 ± 1.7	0.1 ± 0.1	0.6 ± 0.2	n.d.
20:4n-6	0.6 ± 0.0	4.8 ± 0.6	1.2 ± 0.2	5.7 ± 0.3	0.4 ± 0.0
22:4n-6	0.1 ± 0.1	0.3 ± 0.3	1.7 ± 0.1	n.d.	n.d.
22:5n-6	0.1 ± 0.1	1.2 ± 0.1	3.2 ± 0.1	0.2 ± 0.0	n.d.
Total n-6 PUFA	1.2 ± 0.8	11.8 ± 1.4	6.2 ± 0.1	6.5 ± 0.6	0.9 ± 0.1
20:4n-3	0.1 ± 0.1	0.2 ± 0.2	n.d.	0.5 ± 0.0	n.d.
20:5n-3	0.1 ± 0.1	0.3 ± 0.3	0.4 ± 0.3	0.6 ± 0.1	0.4 ± 0.1
22:5n-3	0.1 ± 0.1	0.7 ± 0.1	2.1 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
22:6n-3	0.5 ± 0.0	5.1 ± 0.6	11.0 ± 1.2	0.7 ± 0.1	0.8 ± 0.2
Total n-3 PUFA	0.8 ± 0.2	6.3 ± 0.1	13.5 ± 0.9	1.9 ± 0.1	1.4 ± 0.2
Total PUFA	15.8 ± 1.3	44.8 ± 0.8	39.9 ± 0.6	43.8 ± 0.8	13.4 ± 0.8
Total dimethylacetals	n.d.	1.7 ± 0.5	n.d.	n.d.	0.8 ± 0.1
Total unidentified	2.6 ± 0.1	1.0 ± 1.2	2.8 ± 0.3	2.2 ± 0.3	4.8 ± 1.2

[a]Results are means ± SD (n = 3); n.d., not detected; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TN, total neutral lipids.
[b]Predominantly n-7 isomer.

fatty acid), and medium was present at 267 μL/cm^2 (representing about 200 and 13.5 μg of total fatty acid and PUFA, respectively). Therefore, the medium contained a great excess of fatty acids including PUFA, over the amount of total fatty acids as determined at confluence. Although the precise rates of turnover and consumption of fatty acids by PLHC-1 cells were not determined, it appears unlikely that the medium is deficient in EFA and highly likely that there is sufficient n-6 and n-3 PUFA to satisfy general cell requirements for PUFA. Biochemically, there would appear to be more than enough long-chain n-3 and n-6 PUFA available to effectively inhibit the metabolism of 18:1n-9 *via* the desaturation/elongation pathway (9), but this does not happen in the PLHC-1 cells. Therefore, the production of 22:3n-9 and 22:4n-9 in PLHC-1 cells may be indicative of unusual metabolism which is perhaps a specific feature of PLHC-1 cells.

Some mammalian cell lines have also been shown to contain detectable n-9 PUFA in the absence of EFA-deficiency conditions. The presence of 20:3n-9 has been reported at levels of up to 4% of total fatty acids in total lipid from MDCK cells grown in 10% newborn calf serum (36), total phospholipids from THP-1 cells grown in 10% FCS (37), and lipids from rat liver epithelial cells and primary hepatocytes cultured in 10% FCS (38). Mead acid also accumulated in total phospholipids in confluent cultures of 3T3 cells grown in 10% FCS (39). No C_{22} n-9 PUFA were detected in the cell

lipid in any of these studies. Therefore, PLHC-1 cells were unusual both in the amount of n-9 PUFA produced (>21% of total fatty acids) and the fact that a substantial amount (>5% of total fatty acids) of it was the elongated and desaturated derivatives of 20:3n-9, 22:3, and 22:4.

However, the presence of 22:4n-9 in PLHC-1 cells is not entirely unique as it has some similarity to rat sperm where very long-chain n-9 PUFA have been shown to be formed as a part of normal metabolism (40,41). Mammalian sperm characteristically contains high percentages of C_{22} PUFA, generally 22:5n-6 and 22:6n-3 (42,43), but rat sperm is unique in also containing significant amounts of 22:3n-9 and 22:4n-9 (41). The levels of 22:3n-9 and, especially, 22:4n-9 increased during maturation of the sperm, indicating ongoing metabolism of the n-9 PUFA (41). The rats in that study were healthy, well-fed, and maintained on EFA-sufficient diets, leading the authors to conclude that long-chain n-9 PUFA can be normal, physiological components of certain cells (40). Interestingly, small percentages of 24:4n-9 and 24:5n-9 were also found in the rat sperm, as was 20:3n-9 (40,41).

It is not known if the situation in PLHC-1 cells is analogous to the situation described above in rat sperm. For instance, it is not known if hepatic lipids from normal, well-fed EFA-sufficient topminnows contain long-chain n-9 PUFA. Furthermore, it is not known whether hepatocarcinoma tissue from the topminnow, similar to the tissue that the PLHC-1 cell line was derived from, normally contains n-9 PUFA. Therefore, it is not known if the tissue origin, i.e., a tumor, of the PLHC-1 cell line is related to the unusual fatty acid metabolism found in the cell line. This is an area that requires investigation as it could be of considerable interest if lipid and fatty acid metabolism were altered in tumor cells *in vivo*.

All the fish cell lines we have studied to date produced some n-9 PUFA when grown in 10% FCS (16,44–47). RTG-2 cells, a rainbow trout line, contained as much as 8% total n-9 PUFA, over half of which was 20:2n-9 and turbot (TF) cells produced nearly 14% n-9 PUFA, almost all of which was 18:2n-9 (44). However, none of the other fish cell lines we have studied produced C_{22} n-9 PUFA or as much 20:3n-9 as the PLHC-1 cells used in the present study. The TF cells are known to lack or have very low Δ5 desaturase activity, and RTG-2 cells appear to express a low level of Δ4 desaturation (48). Therefore, it would appear that PLHC-1 cells exhibit a wider range of desaturation activities than the other fish cell lines studied to date.

In the present study, 22:3n-9 and 22:4n-9 showed a very characteristic pattern of distribution among the major phospholipid classes, being particularly concentrated in PS as were all the other C_{22} PUFA. In contrast, the 22:3n-9 and 22:4n-9 of rat sperm were more specifically located in the plasmenylcholine fraction with only very small amounts found in the PS fraction (40,41). The overall fatty acyl distribution in the major phospholipids of PLHC-1 cells, with high 16:0 and lower PUFA in PC, high 18:0 and C_{22} PUFA in PS, high 18:0 and C_{20} PUFA in PI, and generally intermediate levels of saturated and monounsaturated fatty acids and PUFA in PE was similar to the distributions found in other fish cell lines (45–47,49). The very high level of 20:3n-9 in PI in PLHC-1 cells was interesting, especially when combined with the highly specific localization of 20:3n-6 in PE, which was a little surprising. However, in rainbow trout gonad and chum salmon heart cells, 20:2n-9 and 20:3n-9 were also found primarily in PI, although in those studies 20:3n-9 could not be resolved from 20:2n-6 (45,49). In Atlantic salmon and TF cells, which do not produce much 20:3n-9, 20:2n-9 was found in the highest percentages in PI (46,47). However, whereas 20:3n-6 was found primarily in PI in chum salmon heart, rainbow trout gonad, and TF cell lines, it was located to a greater extent in PE than PI in Atlantic salmon cells (45–47,49). Overall therefore, the phospholipid class distribution of PUFA in PLHC-1 cells very clearly showed the apparent association between individual phospholipid classes and specific intermediates/products of the desaturation/elongation pathways that has been reported and discussed previously (46). Specifically PI is associated with the immediate products of Δ5 desaturase, and PS is associated with the elongated and further desaturated derivatives of the immediate Δ5 desaturase products.

In conclusion, the lipids of the hepatic tumor cell line, PLHC-1, from the topminnow (*P. lucida*), contain considerable amounts of long-chain n-9 PUFA, including 22:3n-9 and 22:4n-9, despite culture in serum containing sufficient EFA. The results indicate that long-chain PUFA of the n-9 series can be formed in vertebrate tissue other than in conditions of classical EFA deficiency.

ACKNOWLEDGMENT

The authors gratefully thank Dr. L.E. Hightower for supplying the PLHC-1 cells.

REFERENCES

1. Burr, G.O., and Burr, M.M. (1929) A New Deficiency Disease Produced by the Rigid Exclusion of Fat from the Diet, *J. Biol. Chem. 82*, 345–367.
2. Tinoco, J. (1982) Dietary Requirements and Functions of α-Linolenic Acid in Animals, *Prog. Lipid Res. 21*, 1–46.
3. Vergroesen, A. (ed.) (1975) *The Role of Fats in Human Nutrition*, Academic Press, New York.
4. Sargent, J.R. (1976) The Structure, Metabolism and Function of Lipids in Marine Organisms, *Biochemical and Biophysical Perspectives in Marine Biology* (Malins, D.C., and Sargent, J.R., eds.) Vol. 3, pp. 149–212, Academic Press, New York.
5. Ackman, R.G. (1980) Fish Lipids, Part 1, *Advances in Fish Science and Technology* (Connell, J.J., ed.) pp. 86–103, Fishing News Books Ltd., Farnham, Surrey.
6. Ackman, R.G. (1982) Fatty Acid Composition of Fish Oils, *Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oil* (Barlow, S.M., and Stansby, M.E., eds.) pp. 25–88, Academic Press, London.
7. Holman, R.T. (1970) Essential Fatty Acid Deficiency, *Prog. Chem. Fats and Other Lipids 9*, 279–348.
8. Carroll, K.K. (1965) Dietary Fat and the Fatty Acid Composition of Tissue Lipids, *J. Am. Oil Chem. Soc. 42*, 516–520.

9. Brenner, R.R., and Peluffo, R.O. (1966) Effect of Saturated and Unsaturated Fatty Acids on the Desaturation *in Vitro* of Palmitic, Stearic, Oleic, Linoleic and Linolenic Acids, *J. Biol. Chem. 241*, 5213–5219.
10. Mead, J.F,. and Slaton, W.H. (1956) Metabolism of Essential Fatty Acids. III. Isolation of 5,8,11-Eicosatrienoic Acid from Fat-Deficient Rats, *J. Biol. Chem. 219*, 705–709.
11. Walker, B.L. (1966) Chromatographic Evidence for the Occurrence of Oleic Acid Metabolites in Erythrocytes from Essential Fatty Acid-Deficient Rats, *Arch. Biochem. Biophys. 114*, 465–471.
12. Watanabe, T. (1982) Lipid Nutrition in Fish, *Comp. Biochem. Physiol. 73B*, 3–15.
13. Henderson, R.J., and Tocher, D.R. (1987) The Lipid Composition and Biochemistry of Freshwater Fish, *Prog. Lipid Res. 26*, 281–347.
14. Holman, R.T. (1986) Nutritional and Biochemical Evidences of Acyl Interaction with Respect to Essential Polyunsaturated Fatty Acids, *Prog. Lipid Res. 25*, 29–39.
15. Tocher, D.R., Sargent, J.R., and Frerichs, G.N. (1988) The Fatty Acid Compositions of Established Fish Cell Lines After Long-Term Culture in Mammalian Sera, *Fish Physiol. Biochem. 5*, 219–227.
16. Tocher, D.R., and Dick, J.R. (1990) Polyunsaturated Fatty Acid Metabolism in Cultured Fish Cells: Incorporation and Metabolism of n-3 and n-6 Series Acids by Atlantic Salmon (*Salmo salar*) Cells, *Fish Physiol. Biochem. 8*, 311–319.
17. Ryan, J.A., and Hightower, L.E. (1994) Evaluation of Heavy-Metal Ion Toxicity in Fish Cells Using a Combined Stress Protein and Cytotoxicity Assay, *Environ. Toxicol. Chem. 13*, 1231–1240.
18. Tocher, D.R., Castell, J.D., Dick, J.R., and Sargent, J.R. (1994) Effects of Salinity on the Growth, Lipid Content and Lipid Class Composition of Atlantic Salmon (*Salmo salar*) and Turbot (*Scophthalmus maximus*) Cells in Culture, *Fish Physiol. Biochem. 13*, 451–461.
19. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
20. Tocher, D.R., and Harvie, D.G. (1988) Fatty Acid Compositions of the Major Phosphoglycerides from Fish Neural Tissues; n-3 and n-6 Polyunsaturated Fatty Acids in Rainbow Trout (*Salmo gairdneri*) and Cod (*Gadus morhua*) Brains and Retinas,*Fish Physiol. Biochem. 5*, 229–239.
21. Henderson, R.J., and Tocher, D.R. (1992) Thin-Layer Chromatography, *Lipid Analysis: A Practical Approach* (Hamilton, R.J. and Hamilton, S., eds.) pp. 65–111, IRL Press, Oxford.
22. Olsen, R.E., and Henderson, R.J. (1989) The Rapid Analysis of Neutral and Polar Marine Lipids Using Double-Development HPTLC and Scanning Densitometry, *J. Exp. Mar. Biol. Ecol. 129*, 189–197.
23. Vitiello, F., and Zanetta J.-P. (1978) Thin-Layer Chromatography of Phospholipids, *J. Chromatogr. 166*, 637–640.
24. Christie, W.W. (1982) *Lipid Analysis*, 2nd edn., Pergamon Press, Oxford.
25. Christie, W.W., and Stefanov, K. (1987) Separation of Picolinyl Ester Derivatives of Fatty Acids by High-Performance Liquid Chromatography for Identification by Mass Spectrometry, *J. Chromatogr. 392*, 259–265.
26. Evershed, R.P. (1992) Mass Spectrometry of Lipids, *Lipid Analysis: A Practical Approach* (Hamilton, R.J., and Hamilton, S., eds.) pp. 263–308, IRL Press, Oxford.
27. Cinti, D.L., Cook, L., Nagi, M.N., and Suneja, S.K. (1992) The Fatty Acid Chain Elongation System of Mammalian Endoplasmic Reticulum, *Prog. Lipid Res. 31*, 1–51.
28. Banerjee, N., and Rosenthal, M.D. (1986) Elongation of C_{20} Polyunsaturated Fatty Acids by Human Skin Fibroblasts, *Biochim. Biophys. Acta 878*, 404–411.
29. Stubbs, C.D., and Smith, A.D. (1984) The Modification of Mammalian Membrane Polyunsaturated Fatty Acid Composition in Relation to Membrane Fluidity and Function, *Biochim. Biophys. Acta 779*, 89–137.
30. Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H. (1991) The Metabolism of 7,10,13,16,19-Docosapentaenoic Acid to 4,7,10,13,16,19-Docosahexaenoic Acid in Rat Liver Is Independent of a 4-Desaturase, *J. Biol. Chem. 266*, 19995–20000.
31. Voss, A., Reinhart, M., and Sprecher, H. (1992) Differences in the Interconversion Between 20-Carbon n-3 and n-6 Polyunsaturated Fatty Acids in Rat Liver, *Biochim. Biophys. Acta 1127*, 33–40.
32. Sprecher, H. (1992) A Reevaluation of the Pathway for the Biosynthesis of 4,7,10,13,16,19-Docosahexaenoic Acid, *Omega-3 News 7*, 1–3.
33. Karmiol, S., and Bettger, W.J. (1988) Accumulation of n-9-Eicosatrienoic and Docosatrienoic Acids in Human Fibroblast Phospholipids, *Lipids 23*, 891–898.
34. Karmiol, S., and Bettger, W.J. (1991) Lack of an Association Between Cellular Phospholipid Triene:Tetraene Ratio and Proliferation of Human Skin Fibroblasts in Culture, *J. Nutr. 121*, 595–604.
35. Bettger, W.J., Driscoll, E.R., and Karmiol, S. (1992) Selective Depletion of Nonesterified Fatty Acids in Fetal Bovine Serum-Supplemented Culture Medium by Human Fibroblasts Proliferating in Low-Density Culture, *J. Nutr. Biochem. 3*, 349–352.
36. Lynch, R.D., Locicero, J., and Schneeberger, E.E. (1986) Metabolism and Incorporation into Glycerolipids of Exogenous 18:3n-3 and 18:3n-6 by MDCK Cells, *Lipids 21*, 447–453.
37. Galella, G., Marangoni, F., Rise, P., Colombo, C., Galli, G., and Galli, C. (1993) n-6 and n-3 Fatty Acid Acculmulation in thp-1 Cell Phospholipids, *Biochim. Biophys. Acta 1169*, 280–290.
38. Pepin, D., Chambaz, J., Rissel, M.Y., Guillouzo, A., and Bereziat, G. (1988) Essential Fatty Acid Pattern of Glycerolipids in Rat Hepatocytes in Primary Culture and in Coculture with Rat Liver Epithelial Cells, *Lipids 23*, 784–790.
39. Hyman, B.T., Stoll, L.L., and Spector, A.A. (1981) Accumulation of n-9-Eicosatrienoic Acid in Confluent 3T3-L1 and 3T3 Cells, *J. Biol. Chem. 256*, 8863–8866.
40. Aveldano, M.I., Rotstein, N.P., and Vermouth, N.T. (1992) Occurrence of Long and Very Long Polyenoic Fatty Acids of the n-9 Series in Rat Spermatozoa, *Lipids 27*, 676–680.
41. Aveldano, M.I., Rotstein, N.P,. and Vermouth, N.T. (1992) Lipid Remodelling During Epididymal Maturation of Rat Spermatozoa: Enrichment in Plasmenylcholines Containing Long-Chain Polyenoic Fatty Acids of the n-9 Series, *Biochem. J. 283*, 235–241.
42. Selivonchick, D.P., Schmid, P.C., Natarajan, V., and Schmid, H.H.O. (1980) Structure and Metabolism of Phospholipids in Bovine Epididymal Spermatozoa, *Biochim. Biophys. Acta 618*, 242–254.
43. Nikolopoulou, M., Soucek, D.A., and Vary, J.C. (1985) Changes in the Lipid Content of Boar Sperm Plasma Membranes During Epididymal Maturation, *Biochim. Biophys. Acta 815*, 486–498.
44. Tocher, D.R., Carr, J., and Sargent, J.R. (1989) Polyunsaturated Fatty Acid Metabolism in Fish Cells: Differential Metabolism of n-3 and n-6 Series Acids by Cultured Cells Originating from a Freshwater Teleost Fish and from a Marine Teleost Fish, *Comp. Biochem. Physiol. 94B*, 367–374.
45. Tocher, D.R. (1990) Incorporation and Metabolism of n-3 and n-6 Polyunsaturated Fatty Acids in Phospholipid Classes in Cultured Rainbow Trout (*Salmo gairdneri*) Cells, *Fish Physiol. Biochem. 8*, 239–249.
46. Tocher, D.R., and Dick, J.R. (1990) Incorporation and Metabo-

lism of n-3 and n-6 Polyunsaturated Fatty Acids in Phospholipid Classes in Cultured Atlantic Salmon (*Salmo salar*) Cells, *Comp. Biochem. Physiol. 96B*, 73–79.
47. Tocher, D.R., and Mackinlay, E.E. (1990) Incorporation and Metabolism of n-3 and n-6 Polyunsaturated Fatty Acids in Phospholipid Classes in Cultured Turbot (*Scophthalmus maximus*) Cells, *Fish Physiol. Biochem. 8*, 251–260.
48. Tocher, D.R., and Sargent, J.R. (1990) Effect of Temperature on the Incorporation into Phospholipid Classes and Metabolism *via* Desaturation and Elongation of n-3 and n-6 Polyunsaturated Fatty acids in Fish Cells in Culture, *Lipids 25*, 435–442.
49. Bell, J.G., and Sargent, J.R. (1992) The Incorporation and Metabolism of Polyunsaturated Fatty Acids in Phospholipids of Cultured Cells from Chum Salmon (*Oncorhynchus keta*), *Fish Physiol. Biochem. 10*, 99–109.

[Received October 31, 1994, and in revised form February 14, 1995; Revision accepted March 13, 1995]

The Glycosylceramides of the Nematode *Caenorhabditis elegans* Contain an Unusual, Branched-Chain Sphingoid Base

David J. Chitwood[a,*], William R. Lusby[b], Malcolm J. Thompson[b], Jan P. Kochansky[b] and Oliver W. Howarth[c]

[a]Nematology Laboratory and [b]Insect Neurobiology and Hormone Laboratory, ARS, USDA, Beltsville, Maryland and [c]Department of Chemistry, University of Warwick, Coventry, United Kingdom

ABSTRACT: *Caenorhabditis elegans* was cultured in semi-defined medium containing yeast extract, soy peptone, glucose, hemoglobin, Tween 80, and sitosterol. Monoglycosylceramides were chromatographically purified from nematode extracts. Their structures were elucidated with mass spectrometry, nuclear magnetic resonance spectroscopy, and analysis of methanolysis products of the parent cerebrosides. The glycosylceramides were unusual in that the only long-chain sphingoid base detected was an *iso*-branched compound with a C-4 double bond (i.e., 15-methyl-2-aminohexadec-4-en-1,3-diol). Glucose was the only sugar moiety detected. The fatty acids consisted of a series of primarily straight-chain, saturated, 2-hydroxylated C_{20}–C_{26} acids; some *iso*-branched analogs also occurred. The sphingomyelins of *C. elegans* were also hydrolyzed, and the same *iso*-branched C_{17} compound was the only sphingoid base detected. This is the first structural analysis of a nematode glycosphingolipid and the first report of an organism in which the long-chain sphingoid bases are entirely *iso*-branched.
Lipids 30, 567–573 (1995).

Glycosphingolipids are ubiquitous components of animal cell membranes, where they possess a remarkable diversity of functions (1). Some of these roles are structural, such as providing increased rigidity to membranes or serving as anchors for cellular proteins (1,2). In recent years, intensive research has focused upon the roles of glycosphingolipids as antigenic determinants, mediators of recognition events, and precursors for lipid second messengers involved in signal transduction pathways (3).

Although many nematodes are important parasites of animals and plants, structural analysis of glycosphingolipids from nematodes has not been reported. The lack of direct knowledge of nematode glycosphingolipids is surprising, given that the surfaces of some animal–parasitic nematodes contain glycolipid (4) and that glycoconjugates on the nematode surface are suspected to be involved in chemoreception (5), recognition, and other aspects of host–parasite interaction between parasitic nematodes and their hosts (4,6–8). The only analytical investigations of nematode glycosphingolipids have been thin-layer chromatography (TLC) characterizations (9–14).

During recent years, our research has focused on nematode steroid biochemistry, including the possible existence of ecdysteroids (insect molting hormones) in nematodes (15). In such investigations of a plant–parasitic nematode *(Meloidogyne incognita)*, we detected large amounts of material that migrated during TLC identically to ecdysone (16). Because identification of this material was difficult and because quantities of nematodes were insufficient for unequivalent structural elucidation, we examined the microbivorous nematode *Caenorhabditis elegans* to determine if it contained similar material. Unlike parasitic nematodes, this species is readily propagated in the laboratory and has become an organism of choice for developmental biologists (17). In this report, we describe the structural elucidation of the unknown lipid from *C. elegans* as a series of cerebrosides (i.e., monoglycosylceramides) with a rather unusual long-chain sphingoid base.

MATERIALS AND METHODS

Culture methods. Caenorhabditis elegans strain N2 was propagated at 22°C in a semi-defined aqueous medium containing 30 mg/mL yeast extract (Difco Laboratories, Detroit, MI), 30 mg/mL soy peptone (Sigma Chemical Co., St. Louis, MO), 10 mg/mL glucose (Sigma), 0.5 mg/mL hemoglobin (Sigma), 10 µg/mL sitosterol [98.5% purity by gas–liquid chromatography (GLC); remaining sterol was campesterol], 0.5 mg/mL Tween 80 (Sigma), and 0.2 µL/mL silicone Antifoam C emulsion (Sigma). Yeast extract, glucose, and hemoglobin were extracted with organic solvents to remove endogenous lipid contaminants (18). Nematodes were propagated axenically in

*To whom correspondence should be addressed at USDA, ARS, Nematology Laboratory, Bldg. 011A, Room 153, BARC-West, Beltsville, MD 20705-2350.

Abbreviations: CI/MS, chemical ionization/mass spectroscopy; EI/MS, electron impact/mass spectrometry; EtOH, ethanol; FAME, fatty acid methyl ester; GalCer, galactosylceramide; GC/MS, gas chromatography/mass spectrometry; GlcCer, glucosylceramide; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; LCB, long-chain sphingoid base; MeOH, methanol; TLC, thin-layer chromatography; TMS, trimethylsilyl. Fatty acids are represented by a binumeric system in which the first number refers to the chain length, and the second number refers to the number of double bonds.

Copyright © 1995 by AOCS Press

acrated 3-L cultures in 4-L side-arm flasks, and mixed life stages were isolated and lyophilized (5.1–5.9 g dry weight per harvest) as described previously (18).

Cerebroside extraction and purification. Although lipids from lyophilized nematodes were initially isolated by extraction with $CHCl_3$/methanol (MeOH) (2:1) (19), in most cases nematodes were extracted three times with hexane/isopropanol (3:2) (20). No additional cerebrosides (i.e., the unknown lipid from *C. elegans*) were detected by TLC or HPLC (high-performance liquid chromatography) when the nematode pellet remaining after the hexane/isopropanol extraction was extracted with $CHCl_3$/MeOH (2:1), although some sphingomyelin was detected. The lipids (*ca.* 1,200 mg) were fractionated on a 19-mm i.d. column containing 17.6 g silica (Kieselgel 60, 70–230 mesh; E. Merck, Darmstadt, Germany) previously deactivated with 15% H_2O; compounds were eluted with 400 mL $CHCl_3$, 250 mL each of $CHCl_3$/acetone (3:1, 2:1, 1:2, and 1:3), 250 mL acetone, and 250 mL MeOH. The unknown material emerged in the $CHCl_3$/acetone 1:2 and 1:3 fractions, as indicated by TLC on high-performance silica gel H plates (Merck) developed with $CHCl_3$/ethanol (EtOH) (70:30). (All EtOH used in this investigation contained 5% H_2O.) Further purification was obtained on a 15-mm i.d. column containing 13.0 g of nondeactivated silica; compounds were eluted with 100-mL fractions of 0, 5, 10, 15, 20, and 40% EtOH in $CHCl_3$ and MeOH. The unknown emerged in the 15% EtOH fraction.

Individual cerebrosides were separated from each other by reversed-phase HPLC with a Spectra Physics (San Jose, CA) model SP8700XR solvent delivery system pumping 2.0% water in MeOH through a Shandon (Runcorn, United Kingdom) C_{18} column (ODS-Hypersil, 25 cm × 4.6 mm i.d., 5 µm particles) at a flow rate of 1.0 mL/min at 30°C. Absorbance at 214 nm was monitored with a Waters (Milford, MA) model 441 detector. Quantification was obtained with a Shimadzu (Columbia, MD) C-R3A recording integrator.

Spectroscopic methods. The most abundant cerebroside was analyzed by electron impact (EI) and chemical ionization (CI) mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Mass spectra were obtained with a Finnigan model 4510 instrument (Finnigan MAT, San Jose, CA) connected to an Incos data system; ammonia and methane CI spectra were produced as described (21). Fourier transform ^1H NMR spectra in C_5D_5N were recorded on a Bruker 400-MHz instrument (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) with tetramethylsilane as a reference. An NMR spectrum was also obtained for an authentic compound purified by HPLC from a mixture of galactocerebrosides (Sigma); the purified standard was *N*-2′-hydroxyoctadecanoyl-1-*O*-β-D-galactopyranosyl-4(*E*)-sphingenine. Some cerebroside derivatives (subsequently described) were analyzed by gas chromatography/mass spectrometry (GC/MS) with a 15-m × 0.32-mm J&W (Folsom, CA) DB-1 fused silica capillary column (0.25 µm film).

Methanolysis. Both the cerebroside mixture (i.e., before HPLC) and individual, HPLC-purified cerebrosides were methanolyzed in 3 mL of a solution of concentrated HCl/H_2O/MeOH (43:47:410) at 70°C for 18 h (22). Fatty acid methyl esters (FAMEs) were extracted from the reaction mixture four times with 3 mL hexane; the lower phase was cooled, adjusted to pH 10 with 4.0 M KOH, and extracted with an equal volume of diethyl ether five times to thoroughly remove the long-chain sphingoid bases (LCBs). The aqueous layer, containing methyl glycosides, was dried in a rotary evaporator and stored in MeOH until analysis.

FAME analysis. The combined hexane extracts of the methanolyzate were dried with a rotary evaporator and applied to a silica Sep-Pak previously conditioned with 5 mL $CHCl_3$ and 10 mL hexane; elutions were with 5 mL hexane, 5 mL hexane/diethyl ether (97.5:2.5) (eluate contained FAMEs), 7 mL hexane/ether (9:1) (hydroxylated FAMEs), 5 mL ether, and 5 mL MeOH. Methyl esters were analyzed by GLC in a Varian (Palo Alto, CA) model 3700 gas chromatograph fitted with a J&W on-column injector, a flame-ionization detector, and a 15.0-m × 0.25-mm J&W DB-1 capillary column. The column temperature was held at 130°C for 1 min after injection and then was increased at 15°C/min to a final temperature of 150–255°C, depending upon the chain length and degree of hydroxylation of the particular compounds. Peaks were quantified with a Shimadzu C-R1B recording integrator. Tentative identifications were confirmed by GC/MS.

LCB analysis. The ether extract of the methanolyzate was applied to a silica Sep-Pak (Waters) previously conditioned with 5 mL MeOH and 10 mL $CHCl_3$. Compounds were eluted from the Sep-Pak with 5 mL $CHCl_3$, 7 mL $CHCl_3$/EtOH (9:1), 5 mL $CHCl_3$/EtOH (4:1), 7 mL $CHCl_3$/EtOH (3:2) (containing LCBs), 5 mL $CHCl_3$/EtOH (2:3), and 5 mL MeOH. The purified LCBs were *N*-acetylated in 240 µL MeOH and 60 µL acetic anhydride under nitrogen overnight at room temperature. After removal of the solvents with a stream of nitrogen, the *N*-acetyl LCBs were purified on a silica Sep-Pak conditioned with 10 mL $CHCl_3$ and eluted with 5-mL fractions of 0, 2, 5, 10, 40, and 100% MeOH in $CHCl_3$. The *N*-acetyl LCBs (in the 5% MeOH fraction) were preparatively purified by HPLC in the same system as the parent cerebrosides, except that a mobile phase of 20% H_2O in MeOH was used. For determination of double-bond position, the major peak was subjected to periodate/permanganate oxidation (23). The resulting free fatty acids were methylated at 60°C under nitrogen overnight in 4 mL of MeOH/benzene (3:1) containing 100 µL H_2SO_4. The methyl esters were then extracted by solvent partition with five transfers of hexane across two tubes containing equal volumes of countersaturated MeOH/water (7:3), purified by the Sep-Pak scheme as in the FAME analysis section, and identified by GLC and GC/MS.

In addition, the purified LCBs were *N,O,O*-triacetylated in pyridine/acetic anhydride (1:1) under nitrogen overnight at room temperature. After removal of the solvents under a stream of nitrogen, the triacetates were purified on a silica Sep-Pak identically to the *N*-acetyl LCBs. The LCB triacetates emerged in the 2% MeOH fraction and were analyzed

by GLC similarly to the FAMEs, except the final column temperature was 240°C.

Methyl glycoside analysis. Following the ether partition, the remaining aqueous phase of the methanolyzate was applied to a C_{18} Sep-Pak previously conditioned with 5.0-mL portions of MeOH, water, MeOH, and water. The methyl glycosides were eluted with 10 mL water; 5 mL MeOH was used to strip the Sep-Pak of any apolar material. The methyl glycosides were separated from the KCl acquired during the methanolysis procedure by elution with 100 mL water through a 15-mm i.d. column containing 10.0 g Bio-Rad (Richmond, CA) AG 501-X8(D) mixed bed resin (24). Water was removed with a rotary evaporator, trimethylsilyl (TMS) derivatives of the methyl glycosides were prepared with 100 µL hexamethyldisilazane/trimethylchlorosilane/pyridine (2:1:10) (TriSil; Pierce, Rockford, IL) (25), and the TMS ethers were analyzed by GLC on the DB-1 column as mentioned previously, with a final column temperature of 160°C.

TLC. All chromatographic fractionations and derivatizations were monitored on Silica Gel 60 high-performance plates (Merck) with an appropriate solvent: $CHCl_3$/95% EtOH (7:3) (cerebrosides); $CHCl_3$/MeOH/water (65:25:4) (sphingomyelin and other phospholipids); hexane/diethyl ether/acetic acid (70:30:1) (fatty acids and methyl esters); or $CHCl_3$/MeOH/10 M aqueous ammonia (66:30:4) (LCBs and methyl glycosides), 90:10:0.2 (*N*-acetyl LCBs), or 95:5:0.2 (LCB triacetates). Compounds were detected by heating plates sprayed with 50% H_2SO_4 at 120°C.

Sphingomyelin analysis. For comparative purposes, the long-chain bases from sphingomyelins and cerebrosides of *C. elegans* were compared. Only *C. elegans* extracted with hexane/isopropanol was used; however, TLC indicated that sphingomyelins occurred in the $CHCl_3$/MeOH extraction of the nematode pellet remaining after hexane/isopropanol extraction. Therefore, this $CHCl_3$/MeOH extract was applied to a silica column similar to that used with the original hexane/isopropanol extract, and the combined methanolic eluates were considered to be the crude sphingomyelin fraction, which contained several other phospholipids. This material was then applied to a DEAE-cellulose column (8.66 g in a 19-mm i.d. column) prepared and eluted by the procedure of Rouser *et al.* (26). The sphingomyelins and choline phosphoglycerides, which emerged together from the DEAE-cellulose column, were then separated by preparative TLC on Silica Gel 60 chromatoplates pre-developed with the solvent used for fractionation, i.e., $CHCl_3$/MeOH/water 65:25:4. Compounds were extracted from the silica gel with $CHCl_3$/MeOH (2:1) and then hydrolyzed identically to the cerebrosides.

Cerebroside content of medium. Ingredients for 100 mL medium were dissolved in 75 mL water, and 175 mL MeOH was added. Putative cerebrosides were isolated by extraction with two 250-mL portions of hexane. The pooled hexane extracts were fractionated on a silica Sep-Pak conditioned with 5 mL $CHCl_3$; lipids were eluted with 5-mL portions of 0, 5, 10, and 30% EtOH in $CHCl_3$ and MeOH. Putative cerebrosides in the 30% EtOH fraction were analyzed with the same HPLC system used for the *C. elegans* cerebrosides.

Analytical standards. Cerebroside, ceramide, phospholipid, sphingosine (4-sphingenine), and methyl glycoside standards were from Sigma; *iso-* and *anteiso*-FAME standards were from Matreya (Pleasant Gap, PA); and ecdysone was from Simes Pharmaceuticals (Milan, Italy). Hydroxylated FAME standards were prepared from a methylated mixture of fatty acids [Humko (Memphis, TN) Hystrene 9022] by oxygenation of the lithium diisopropylamide-generated dianions (27).

RESULTS

The 15% ethanolic eluate of the silica column contained TLC-homogeneous material which migrated slightly faster than authentic ecdysone. (After the unknown had been partially characterized, it was found to migrate between bovine brain galactosylceramides containing hydroxylated and nonhydroxylated fatty acids.) Reversed-phase HPLC of this material produced over a dozen distinct peaks (Fig. 1).

NH_3 CI/MS of the major HPLC peak (Fig. 1, peak 6) [*m/z* (relative intensity): 803 ($M + NH_4$, 100), 786 ($M + H$, 21), 768 ($M + H - H_2O$, 17)] indicated that the molecular weight of the unknown was 785. A weak ion at *m/z* 641 ($M + NH_4 - 162$) suggested loss of a hexose moiety. Presence of a base peak at 804 in $^{15}NH_3$ CI/MS confirmed that the peak at 803 in the NH_3 spectrum had only one adducted nitrogen atom.

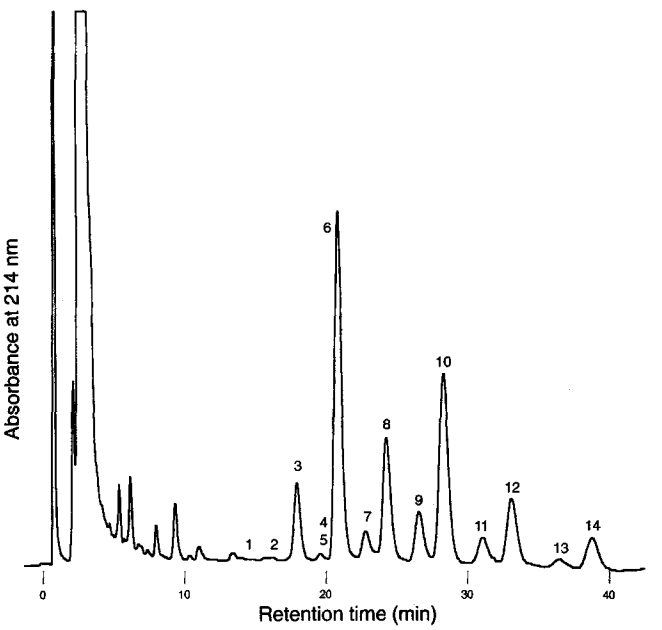


FIG. 1. High-performance liquid chromatography chromatogram of glucosylceramides of *Caenorhabditis elegans*. Glucosylceramides were fractionated on a Shandon (Runcorn, United Kingdom) ODS-Hypersil C_{18} column (25 cm × 4.6 mm i.d., 5 µm particles) with 1.0 mL/min of 2.0% H_2O in methanol at 30°C. Absorbance at 214 nm was monitored with a Waters (Milford, MA) model 441 detector.

Methane CI/MS data [814 (M + C_2H_5, 14), 786 (M + H) 100, 768 (M + H – H_2O, 23), 624 (M + H – 162, 14)] was consistent with the NH_3 data. Finally, the ND_3 CI/MS [814 (100), 794 (18), 774 (14)] indicated the presence of seven exchangeable hydrogen atoms on the parent molecule.

Attempted methylation of the major HPLC-purified component with diazomethane failed to alter the TLC migration of the compound, indicating the lack of a carboxylic acid functional group. Attempted weak acid hydrolysis of the unknown yielded several products. TLC revealed that an apolar product moved identically to a 2-hydroxy FAME standard (methyl 2-hydroxydocos-13-enoate) and that a highly polar, suspected hexose-like product moved identically to an α-methyl galactoside standard. The TLC results, the number of exchangeable hydrogens, and the odd molecular weight of the parent compound indicated the possibility of a glycosphingolipid; therefore, a methanolysis procedure suitable for glycosphingolipids was employed. This methanolysis yielded three products of greatly different polarities, as judged by TLC. The most apolar of these products (i.e., from the hexane extraction of the reaction mixture) yielded MS data from NH_3 CI/MS [405 (M + $(NH_3)_2H$, 13), 388 (M + NH_4, 100)] and ND_3 CI/MS [413 (12), 393 (100)] indicating that the product had a molecular weight of 370 and one exchangeable hydrogen. The EI/MS data [370 (M^+, 25), 352 (M–H_2O, 1), 338 (M – CH_3OH, 3), 311 (M – CH_3COO), 292 (M – $HCOOCH_3$ – H_2O, 6), 57 (C_4H_9, 100)] and GLC retention time were identical with those from a 2-hydroxydocosanoic acid methyl ester standard.

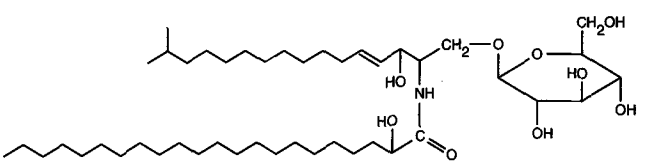
The compound purified from the ether extraction of the methanolyzate of the major HPLC fraction had a molecular weight of 285, as indicated by CI/MS data obtained with NH_3 [303, (M + NH_4, 100), 286 (M + H, 67)] and CH_4 [286 (M + H, 100), 268 (M + H – H_2O, 42)]. EI/MS data revealed little structural information, except for a loss of CH_2OH to produce a weakly abundant (2% relative to the base peak at *m/z* 60) ion at *m/z* 254. Because TLC indicated co-migration with sphing-4-enine, a C-17 sphingoid structure was suspected. The *gem*-dimethyl obtained in the NMR spectrum of the parent cerebroside and the lack of such a dimethyl in the fatty acid moiety of the molecule suggested an *iso*-branched structure for the sphingoid base. Following *N*-acetylation of the sphingoid base, HPLC purification of the *N*-acetyl LCB, permanganate/periodate oxidation of the *N*-acetyl LCB and methylation of the oxidation products, a single peak was obtained in GLC that migrated identically to an *iso*-13:0 FAME. This established that the sphingoid base was indeed an *iso*-17:1 compound, i.e., 15-methyl-2-aminohexadec-4-en-1,3-diol.

The molecular weight of the product obtained from the aqueous phase of the extracted methanolyzate was 194 with 4 exchangeable hydrogens, based on CI/MS data obtained with NH_3 [229 (M + $(NH_3)_2H$, 100), 212 (M + NH_4, 68)], $^{15}NH_3$ [231 (100), 213 (79)], and ND_3 [240 (100), 220 (43)]. Following trimethylsilylation of the material, GLC detected two peaks with retention times identical to those of TMS ethers of α- and β-methyl glucosides in a 2.2:1 ratio. The EI/MS of the peaks were identical to those of TMS ethers of α- or β-methyl glucoside, galactoside, or mannoside, all of which were differentiated poorly by MS, but resolved excellently by GLC; GLC revealed a 76:24 mixture of α- and β-methyl glucoside TMS ethers.

The results obtained from ^1H NMR analysis were consistent with those obtained from analysis of the hydrolysis products. The most striking feature of the NMR spectrum of the major, HPLC-purified compound was a strong doublet at δ0.651 ppm (6H, J = 6.68 Hz), indicative of *gem*-dimethyl moiety in the compound. This doublet was absent in the spectrum of the reference cerebroside. A broad peak at δ1.04 ppm occurred in the methylene region. A septet (J = 6.66 Hz) centered at δ1.27 ppm represented the single tertiary hydrogen between the *gem*-dimethyls. Other features of the NMR spectrum included singlets at δ1.50 and δ1.60 ppm (also present in the NMR of the standard), representing the hydrogens at C-3 of the 2-hydroxydocosanoic acid moiety (28), a quartet (identical to that in the standard) at δ1.86 (J = 7.28 Hz) from the C-6 hydrogens on the sphingoid base (28,29), a sugar region (δ3.4–5.8 ppm) greatly different from the galactosylceramide (GalCer) standard, two double doublets at δ5.69 and δ5.76 ppm (J = 15.36 Hz), representing the two vinylic hydrogens and indicative of an *E*-double bond (28,29), and a doublet at δ7.49 ppm (J =5.16 Hz), representing the amino hydrogen (30). Unfortunately, although the reference GalCer contained a well-defined doublet at δ4.01 ppm (J = 6.8 ppm) indicative of a β-configuration at C-1 of the galactose (28), the region from δ4.16–4.28 [where a doublet (J = 7–9 Hz) would be indicative of a β-conformation at C-1 of the glucose moiety (29–31)] consisted of a pentet at δ4.17 ppm. This multiplet, plus the lack of the expected doublet for an α-conformation at δ4.86 ppm (J = 3.4 Hz) (29–31) indicates that the GluCer (Scheme 1) from *C. elegans* likely has the β-conformation.

After the material had been conclusively identified as a series of glucosylceramides, the HPLC-purified material was quantified by ultraviolet absorbance. Because a source for GluCer standards containing hydroxylated fatty acids could not be located, the basis for quantification was the average absorbance during HPLC for the fatty acid-hydroxylated, bovine brain galactocerebrosides, and the nonhydroxylated, human (Gaucher's) spleen glucocerebrosides. Quantification indicated that the glucosylceramides comprised 0.17% of the dry weight of the nematodes.

After hydrolysis of the most abundant compounds, the material in each remaining HPLC peak was hydrolyzed. In each case, the methyl glycosides were identified with GLC of the TMS ethers as methyl glucosides, the sphingoid base was



SCHEME 1

TABLE 1
Quantification of Glucosylceramides of *Caenorhabditis elegans*, as Determined by Absorbance at 214 nm During High-Performance Liquid Chromatography (HPLC)[a]

Peak number[b]	2-Hydroxy fatty acid[c]	ECL	Relative percentage
1	20:0	21.23	0.5
2	*iso*-21:0	21.87	0.7
3	21:0	22.23	6.8
4	*iso*-22:0	22.87	0.6
5	22:1	22.98	0.1
6	22:0	23.23	31.6
7	*iso*-23:0	23.87	3.8
8	23:0	24.24	12.5
9	*iso*-24:0	24.87	6.0
10	24:0	25.24	21.0
11	*iso*-25:0	25.88	3.4
12	25:0	26.25	7.9
13	*iso*-26:0	26.87	1.6
14	26:0	27.25	3.5

[a]The sugar and sphingosine moieties of all glycosylceramides were determined as glucose and 15-methyl-2-aminohexadec-4-en-1,3-diol with procedures described in the Materials and Methods section.
[b]Peaks refer to the chromatogram depicted in Figure 1.
[c]Fatty acids were identified by gas chromatography/mass spectrometry of the methanolysis products of the HPLC-purified peaks. The equivalent chain lengths (ECLs) of the fatty acid methyl esters, all of which were 2-hydroxylated, were obtained on a J&W (Folsom, CA) DB-1 capillary column and were calculated with respect to nonhydroxylated fatty acid methyl ester standards. Fatty acids are represented by a binumeric system in which the first number refers to the chain length and the second number refers to the number of double bonds.

identified by GLC of the LCB triacetates as 15-methyl-2-aminohexadec-4-en-1,3-diol, and the fatty acids were identified by GC/MS as the compounds shown in Table 1. Although hydroxylated *iso*-fatty acid standards were unavailable, comparison of GLC results of authentic nonhydroxylated *n*-, *iso*-, and *anteiso*-FAME standards indicated that the *iso* and *anteiso* compounds (which did achieve baseline separation) emerged 0.36 and 0.29 units, respectively, before the *n*-isomers. Because the methyl esters of the hydroxylated, branched fatty acids from *C. elegans* had equivalent chain lengths 0.36 or 0.37 units lower than the corresponding hydroxylated *n*-FAMEs, we concluded that the branched methyl esters were *iso*.

Sphingomyelin LCB. Acid hydrolysis of sphingomyelin from *C. elegans* yielded a single peak in the ether extract of the hydrolysis products. The purified *N*-acetyl and triacetyl derivatives of this compound co-migrated during HPLC and GLC, respectively, with the respective *N*-acetyl LCB and triacetate of the sphingoid base isolated from the cerebrosides of *C. elegans*. Therefore, the sphingoid base in the sphingomyelin from *C. elegans* was also concluded to be 15-methyl-2-aminohexadec-4(*E*)-en-1,3-diol.

Cerebroside content of medium. Most of the material in the $CHCl_3$/EtOH (7:3) Sep-Pak fraction generated from 100 mL medium ingredients emerged near the solvent front during HPLC, well in advance of the cerebroside elution volume. The polar nature of this fraction undoubtedly resulted from its cruder purification relative to the cerebrosides from *C. elegans*. Three small peaks (containing 5.9 μg total) were detected in the cerebroside region; however, their retention times did not correspond to any of the retention times for the cerebrosides from *C. elegans*. Because of the minuscule amounts of this material and lack of correlation of its retention times with those of the major cerebrosides of *C. elegans*, we considered identification of this material to be beyond the scope of this investigation.

DISCUSSION

The identification of the unknown material from *C. elegans* as a mixture of cerebrosides containing a homologous series of fatty acids was interesting from several perspectives. Perhaps the most remarkable structural feature was the detection of 15-methyl-2-aminohexadec-4(*E*)-en-1,3-diol as the only sphingoid base. Branched LCBs do occur in other organisms, including mammals, bacteria, protozoa, mollusks, starfish, and a sponge (32–36). Rarely, however, have such isomers been reported as the predominant sphingoid bases; the only other reported occurrence to our knowledge is in the sponge *Halichondria japonica*, in which an *anteiso*-, trihydroxy, saturated C_{19} compound is the major (70%) sphingoid base. The lack of detectable amounts of sphingoid bases other than an *iso*-compound seems unique to *C. elegans* at this point.

The homogeneity of both the sugar and sphingoid base compositions was unexpected. The cerebrosides of most organisms, including most other species of invertebrates examined to date, contain both GluGer and GalCer (37–39). The presence of hydroxylated C_{22} and C_{24} fatty acids is a common feature of many mammalian and invertebrate cerebrosides (37,38). Although *iso*-fatty acids are not necessarily typical of cerebrosides, the glycerolipids of *C. elegans* (40) and some other nematodes (41,42) do contain substantial quantities of shorter (C_{15-18}) *iso*-fatty acids. A notable feature was the presence of only a minute amount of unsaturation, in contrast to the large degree of unsaturation in the glycerolipids of *C. elegans* (40).

Failure to detect cerebrosides in the medium used for propagation of *C. elegans* indicates that the nematodes could biosynthesize cerebrosides from simpler precursors. Although complex glycosphingolipids (such as lactosylceramide or gangliosides) could exist in the medium components and be degraded by *C. elegans*, this possibility seems unlikely because of the branched structure of the nematode LCB and detection of only straight-chain LCBs in GlcCer from soybean (43) and in glycosphingolipids from yeast (44), the latter which contain phosphoinositol as the polar head group.

Although the glycosphingolipids of cestodes and trematodes have been the subject of recent research interest (38,39,45–48), this is the first report of the detailed structural analysis of a nematode glycosphingolipid other than by TLC (9–14). Interestingly, GluCer occurred in much lower concentration than GalCer in adults of the bovine parasite *Onchocerca gibsoni* (9), and in the only TLC study in which cerebrosides with different sugars were spotted on TLC chro-

matoplates. In our research, a faintly visible spot did appear near a hydroxylated GalCer standard when *C. elegans* polar lipids from another silica column fraction were spotted; therefore, *C. elegans* may contain some other hexosylceramide, but in a much lesser quantity than GluCer.

Although sphingosine and sphinganine have been characterized radiochemically in a TLC investigation of *Ascaridia galli* (49), our report is the first chemical analysis of the sphingoid base from nematode sphingomyelin. Interestingly, the only detected sphingoid base from *C. elegans* sphingomyelin is the same compound as was present in the glycosylceramides, i.e., 15-methyl-2-aminohexadec-4(*E*)-en-1,3-diol. The branched structure of the *C. elegans* sphingoid base warrants structural determination of the sphingoid bases of other nematodes to determine if this peculiarity is common to the phylum.

The large amount of the glucocerebroside in *C. elegans* could indicate that the compound has a structural role in stabilizing cellular membranes (50). Because of the growing realization that various parts of the glycosphingolipid molecule (e.g., sphingosine, ceramide) are released and can have important roles as cellular messengers (3), a similar role for such compounds in nematodes or in the interactions of nematodes with their hosts is likely. A possible involvement of glycosphingolipids in host–parasite relationships and a potential for such compounds to be targets for potentially antinematodal compounds have been proposed (9). Scott *et al.* (4) found a 6,000–10,000 Da surface-associated glycolipid in *Dirofilaria immitis* that may be associated with evasion of the host immune system or may be involved in peptide anchoring. Although the compound from *D. immitis* may not be a glycosphingolipid, the importance of recognition events in host–parasite relationships of both animal– and plant–parasitic nematodes indicates that structural elucidation of glycosphingolipids more complex than the rather simple molecules described herein should indeed prove interesting.

ACKNOWLEDGMENTS

We thank Patricia Oakley and Dawn Harrison for skilled technical assistance. The *Caenorhabditis elegans* N2 isolate used in this investigation was originally supplied by the Caenorhabditis Genetics Center, which is supported by the National Institutes of Health.

REFERENCES

1. Merrill, A.H., Jr., Hannun, Y.A., and Bell, R.M. (1993) *Adv. Lipid Res. 25*, 1–24.
2. Hakomori, S. (1981) *Ann. Rev. Biochem. 50*, 733–764.
3. Bell, R.M., Hannun, Y.A., and Merrill, A.H., Jr., (eds.) (1993) *Sphingolipids Part A: Functions and Breakdown Products: Advances in Lipid Research, Vol. 25*, Academic Press, San Diego.
4. Scott, A.L., Diala, C., Moraga, D.A., Ibrahim, M.S., Redding, L., and Tamashiro, W.K. (1988) *Exp. Parasitol. 67*, 307–323.
5. Robertson, W.M., Spiegel, Y., Jansson, H.-B., Marban-Mendoza, N., and Zuckerman, B.M. (1989) *Nematologica 35*, 180–186.
6. Davis, E.L., Kaplan, D.T., Permar, T.A., Dickson, D.W., and Mitchell, D.J. (1988) *J. Nematol. 20*, 609–619.
7. Apfel, H., Eisenbeiss, W.F., and Meyer, T.F. (1992) *Mol. Biochem. Parasitol. 52*, 63–74.
8. Hill, D.E., Fetterer, R.H., and Urban, J.F., Jr. (1991) *Exp. Parasitol. 73*, 376–383.
9. Maloney, M.D., and Semprevivo, L.H. (1991) *Parasitol. Res. 77*, 294–300.
10. Kapur, J., and Sood, M.L. (1985) *Zbl. Vet. Med. 32B*, 345–353.
11. Sarwal, R., Sanyal, S.N., and Khera, S. (1989) *J. Helminthol. 63*, 287–297.
12. Vykhrestyuk, N.P., Yarygina, G.V., and Andreeva, L.I. (1974) *J. Evol. Biochem. Physiol. 10*, 134–137.
13. Kwong, A.Y.H., Wong, P.C.L., and Ko, R.C. (1990) *Comp. Biochem. Physiol. 95B*, 193–197.
14. Aggarwal, R., Sanyal, S.N., and Khera, S. (1989) *Acta Vet. Hung. 37*, 335–347.
15. Chitwood, D.J. (1991) in *Physiology and Biochemistry of Sterols* (Patterson, G.W., and Nes, W.D., eds.) pp. 257–293, American Oil Chemists' Society, Champaign.
16. Chitwood, D.J., McClure, M.A., Feldlaufer, M.F., Lusby, W.R., and Oliver, J.E. (1987) *J. Nematol. 19*, 352–360.
17. Wood, W.B. (ed.) (1988) *The Nematode Caenorhabditis elegans*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
18. Chitwood, D.J., and Feldlaufer, M.F. (1990) *J. Nematol. 22*, 598–607.
19. Folch, J., Lees, M., and Stanley, G.H.S. (1957) *J. Biol. Chem. 226*, 497–509.
20. Hara, A., and Radin, N.S. (1978) *Anal. Biochem. 90*, 420–426.
21. Lusby, W.R., Oliver, J.E., and Thompson, M J. (1987) in *Applications of New Mass Spectrometry Techniques in Pesticide Chemistry* (Rosen, J.D., ed.) pp. 99–115, John Wiley & Sons, New York.
22. Gaver, R.C., and Sweeley, C.C. (1965) *J. Am. Oil Chem. Soc. 42*, 294–298.
23. Hayashi, A., and Matsubara, T. (1971) *Biochim. Biophys. Acta 248*, 306–314.
24. Thompson, M.J., Feldlaufer, M.F., Lozano, R., Rees, H.H., Lusby, W.R., Svoboda, J.A., and Wilzer, K.R. (1987) *Arch. Insect Biochem. Physiol. 4*, 1–15.
25. Sweeley, C.C., Bentley, R., Makita, M., and Wells, W.W. (1963) *J. Am. Oil Chem. Soc. 85*, 2497–2507.
26. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967) in *Lipid Chromatographic Analysis* (Marinetti, G.V., ed.) pp. 99–162, Marcel Dekker, New York.
27. Muralidharan, F.N., and Muralidharan, V.B. (1984) *Chem. Phys. Lipids 34*, 257–263.
28. Dabrowski, J., Egge, H., and Hanfland, P. (1980) *Chem. Phys. Lipids 26*, 187–196.
29. Kawai, G. (1989) *Biochim. Biophys. Acta 1001*, 185–190.
30. Sitrin, R.D., Chan, G., Dingerdissen, J., DeBrosse, C., Mehta, R., Roberts, G., Rottschaefer, S., Staiger, D., Valenta, J., Snader, K.M., Stedman, R.J., and Hoover, J.R.E. (1988) *J. Antibiotics 41*, 469–840.
31. Dabrowski, J., Hanfland, P., and Egge, H. (1982) *Meth. Enzymol. 83*, 69–86.
32. Karlsson, K.-A. (1970) *Lipids 5*, 878–891.
33. Sugita, M., Itasaka, O., and Hori, T. (1976) *Chem. Phys. Lipids 16*, 1–8.
34. Yasugi, E., Kasama, T., and Seyama, Y. (1988) *J. Biochem. 103*, 889–893.
35. Irie, A., Kubo, H., and Hoshi, M. (1990) *J. Biochem. 107*, 578–586.
36. Hayashi, A., Nishimura, Y., and Matsubara, T. (1991) *Biochim. Biophys. Acta 1083*, 179–186.
37. Gurr, M.I., and Harwood, J.L. (1991) *Lipid Biochemistry*, 4th edn., Chapman and Hall, London.
38. Hori, T., and Sugita, M. (1991) *Prog. Lipid Res. 32*, 25–45.

39. Dennis, R.D., and Wiegandt, H. (1993) *Adv. Lipid Res. 26*, 321–351.
40. Hutzell, P.A., and Krusberg, L.R. (1982) *Comp. Biochem. Physiol. 73B*, 517–520.
41. Krusberg, L.R. (1972) *Comp. Biochem. Physiol. 41B*, 89–98.
42. Chitwood, D.J., and Krusberg, L.R. (1981) *Comp. Biochem. Physiol. 69B*, 115–120.
43. Shibuya, H., Kawashima, K., Sakagami, M., Kawanishi, H., Shimomura, M., Ohashi, K., and Kitagawa, I. (1990) *Chem. Pharm. Bull. 38*, 2933–2938.
44. Lester, R.L., Wells, G.B., Oxford, G., and Dickson, R.C. (1993) *J. Biol. Chem.* 268, 845–856.
45. Nishimura, K., Suzuki, A., and Kino, H. (1991) *Biochim. Biophys. Acta 1086*, 141–150.
46. Makaaru, C.K., Damian, R.T., Smith, D.F., and Cummings, R.D. (1992) *J. Biol. Chem. 267*, 2251–2257.
47. Persat, F., Bouhours, J.-F., Mojon, M., and Petavy, A.-F. (1992) *J. Biol. Chem. 267*, 8764–8769.
48. Kawakami, Y., Nakamura, K., Kojima, H., Suzuki, M., Inagaki, F., Suzuki, A., Sonoki, S., Uchida, A., Murata, Y., and Tamai, Y. (1993) *J. Biochem. 114*, 677–683.
49. Bankov, I., and Barrett, J. (1993) *Int. J. Parasitol. 23*, 1083–1085.
50. Curatolo, W. (1987) *Biochim. Biophys. Acta 906*, 137–160.

[Received October 24, 1994, and in revised form February 15, 1995; Revision accepted March 22, 1995]

Effects of Dietary Oil Related to the Toxic Oil Syndrome on the Lipids of Guinea Pig Liver Microsomes

E. de la Casa, N. Pérez-González, C. Sánchez-Bernal and M. Llanillo*

Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Edificio Departamental, 37007 Salamanca, Spain

ABSTRACT: The potential effects of oil specimens related to cases of toxic oil syndrome (TOS) on the liver microsomal lipid composition from guinea pigs were investigated. For four weeks, animals were fed diets supplemented with either "case oil" (oil related to cases of TOS) or "control oil" (oil unrelated to cases of TOS), either previously heated or not. Results were compared with those from guinea pigs fed the same diet with no oil. The administration of case oil produced changes in liver microsomal lipid composition. Statistically significant differences were also found between heated case and heated control oils. The cholesterol/phospholipid molar ratios and the major phospholipid class distribution were unaffected under these diet conditions. However, increases in the relative contents of linoleic and arachidonic acids and, simultaneously, a reduction in palmitic and palmitoleic acid levels were observed by diet effects. Heated oil administration decreased the saturated/unsaturated ratios in all cases. Our data suggest that changes observed in the fatty acid composition are attributable to the free fatty acid contents of administered oils. The toxic constituents of case oil seem to be able to alter the liver microsomal lipid composition.

Lipids 30, 575–579 (1995).

"Toxic oil syndrome" (TOS) is an illness that appeared in Spain in May 1981, as a massive epidemic affecting thousands of people (1). The disease has been epidemiologically linked to the ingestion of cooking oil containing rapeseed oil denatured with 2% of aniline (2,3). Although the acute and chronic phases of TOS have been well defined (1,4), the causal agent in the oil has not yet been identified (4,5). Fatty acyl anilides have been proposed as the main toxic agents in the aniline-denatured rapeseed oil that causes TOS (2,3). More recently, several authors have reported evidence pointing to the existence in humans of an association between increased risk of illness and the presence of aniline and anilide contaminants (4,6). Clinical or biological evidence of liver injury has been documented in many patients hospitalized with acute manifestations of TOS (7,8). It is also well established that the oxidative metabolism of drugs and xenobiotics, taking place in liver endoplasmic reticulum, depends on the tissue fatty acid composition and on dietary fat (9,10), and that the microsomal phospholipid composition can be altered by dietary lipid treatments (11–13).

The present study was designed to obtain information about the potential effects of an oil related to cases of TOS on the fatty acid profile of total lipids, as well as on cholesterol and phospholipid levels in liver microsomes from guinea pigs.

MATERIALS AND METHODS

Oil samples. The oil samples for the experiments were provided by the FIS (Fondo de Investigación Sanitaria, Ministerio de Sanidad y Consumo, Madrid, Spain). As indicated by the supplier, samples were coded as "case oil" (specimens from families whose clinical histories met a strict case definition of TOS) and "control oil" (oils from families who did not have TOS) (6). Aliquots of each oil sample heated at 190°C for 30 min are being referred to as "heated oils."

Animals and treatment schemes. Male guinea pigs of the New Zealand strain (Charles River, Lyon, France) weighing 250–400 g were randomly divided into five groups of eight animals each and were fed a basal guinea pig diet (COBAYO 114; PANLAB S.L., Barcelona, Spain) consisting of: protein (18.3%); fats (2.7%); cellulose (11.9%); ashes (8.3%); starch (21.3%); calcium (1.1%); phosphorus (0.6%); sodium (0.25%); moisture (9.9%); lysine (0.95%); methionine (0.3%); vitamin E (24.3%); vitamin A (15 000 I.U./kg diet); and vitamin D (2 000 I.U./kg diet). Animals had free access to food and water. Four groups of animals were fed the same basal diet supplemented with 2 mL/kg body weight/day of case oil or control oil delivered by an esophageal tube over a period of four weeks. One group, referred to as the control group, was kept pair-fed with the other four groups, but received no oil. The treatment groups were: Group 1, consisting of animals fed nonheated control oil; Group 2, consisting of animals fed heated control oil; Group 3, consisting of animals fed nonheated case oil; and Group 4, consisting of animals fed heated case oil.

Preparation of liver microsomal fraction. After fasting, guinea pigs were killed 24 h after the treatments were com-

*To whom correspondence should be addressed at Departamento de Bioquímica y Biología Molecular, Edificio Departamental, Avda. Compo Charro s/n, 37007 Salamanca, Spain.

Abbreviations: MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TOS, toxic oil syndrome; UFA, total unsaturated fatty acid.

Copyright © 1995 by AOCS Press

pleted. Liver specimens were rapidly removed after blood had been drawn from the abdominal aorta, with the animals under anesthesia. The livers were then carefully washed with 0.9% NaCl, weighed, and stored at –80°C until use. Microsomes were obtained as reported by Lake (14) using precooled homogenization Buffer I, containing 50 mM Tris-HCl, 0.154 mM KCl, 1 mM EDTA, pH 7.4. The microsomal pellets were resuspended in Buffer I, their protein contents determined by the method of Lowry *et al.* (15) using bovine serum albumin as the standard, and were stored at –80°C under N_2 until use.

Lipid extraction and lipid class determinations. Total lipids from microsomes were extracted according to the method of Bligh and Dyer (16). The chloroform layers containing the lipids were washed with a 0.73% NaCl solution and evaporated to complete dryness. The final lipid residues were determined gravimetrically and stored under nitrogen at –20°C until analysis.

Total cholesterol of the lipid extracts was evaluated as reported by Kates (17), using free cholesterol as standard. Total phospholipid contents were determined according to Rouser *et al.* (18) by measuring lipid phosphorus after $HClO_4$ treatment. Phospholipid classes were fractionated by two-dimensional thin-layer chromatography on silica gel 60G plates (10 × 20 cm, 0.25 mm layer; Merck, Barcelona, Spain) and identified as reported by Sánchez-Yagüe and Llanillo (19). Phospholipid classes were determined by measuring lipid phosphorus (18).

Fatty acid analysis. Aliquots of lipid extracts from each sample were transmethylated using BF_3/methanol reagent (Supelco, Barcelona, Spain) according to Metcalfe *et al.* (20). The fatty acid methyl esters obtained from the total lipid extracts were analyzed with a Konik-3000 gas chromatograph (Konik, Barcelona, Spain) equipped with a fused silica capillary column Supelcowax 10 (Supelco), 30 m × 0.53 mm, with 1.0-nm film thickness, and a flame-ionization detector. Hydrogen was the carrier gas. The oven was programmed at 190°C for 7 min, then heated from 190–230°C at 2°C/min, and finally held isothermally at 230°C for 13 min. Peaks were identified by comparison of their retention times with those of known fatty acid methyl ester standards (Supelco). Peak areas were measured with an electronic integrator (Konik).

Statistical analysis. Results were treated statistically by analysis of variance. The Scheffé test was used for comparisons among groups. P values ≤0.01 were considered significant.

RESULTS AND DISCUSSION

The compositions of fatty acids and of other constituents of the oil specimens used in the present study have been described by Kilbourne *et al.* (6). The case oil contained relatively high levels of aniline and fatty acid anilides. Linoleic acid was the main fatty acid component in the control oil, whereas linoleic acid was lower in the case oil.

Table 1 shows the microsomal levels of total lipids (μg/mg protein), cholesterol (nmol/mg protein), total phospholipids (nmol/mg protein), and the cholesterol/phospholipid molar ratios measured. There were increases in each lipid class in

TABLE 1
Lipid Composition of Liver Microsomes from Guinea Pigs Fed Either Case or Control Oil[a]

Lipid classes	Control (no oil)	Control oil		Case oil	
		Nonheated	Heated	Nonheated	Heated
Total lipids (μg/mg protein)	70 ± 10[b]	129 ± 10[b,c]	81 ± 13[b]	189 ± 12[c]	194 ± 22[c]
Cholesterol (C) (nmol/mg protein)	24 ± 4[b]	37 ± 5[b,c]	23 ± 6[b]	51 ± 4[c]	51 ± 5[c]
Total phospholipids (PL) (nmol/mg prot.)	71 ± 8[b]	143 ± 12[b,c]	92 ± 15[b]	204 ± 17[c]	224 ± 26[c]
PL classes (% of total)					
Phosphatidylcholine	50.3 ± 0.4	53.1 ± 0.9	52.2 ± 1.2	51.6 ± 0.3	51.3 ± 0.4
Phosphatidylethanolamine	31.2 ± 0.6	30.7 ± 1.1	32.4 ± 0.9	27.9 ± 0.7	31.7 ± 0.5
Phosphatidylinositol + phosphatidylserine	11.5 ± 0.4	11.9 ± 0.8	9.4 ± 1.2	13.5 ± 0.3	10.7 ± 0.4
Phosphatidylglycerol	3.1 ± 0.3	3.3 ± 0.2	3.4 ± 0.7	3.1 ± 0.2	3.2 ± 0.5
Sphingomyelin	2.5 ± 0.3	2.1 ± 0.2	1.8 ± 0.3	2.7 ± 0.2	1.7 ± 0.3
Lysophosphatidylethanolamine	0.7 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.6 ± 0.1	0.8 ± 0.3
C/PL ratio	0.26 ± 0.01	0.23 ± 0.01	0.20 ± 0.02	0.25 ± 0.01	0.23 ± 0.01

[a]Values are means ± SE for eight animals per dietary group. Total lipids were determined gravimetrically. Proteins, lipid phosphorus, and cholesterol were assayed calorimetrically (see Materials and Methods section). The phospholipid contents were calculated by lipid phosphorus determination. Phospholipid class values are given as percentages of total lipid phosphorus in each sample. Comparisons between control and control oil indicate the diet effect and between control oil and case oil indicate the case oil effect.

[b,c]By rows, values with different superscripts are significantly different at $P < 0.01$.

animals fed case oil as compared to the animals fed no oil and those fed the control oil. Statistically significant differences were found between heated case and heated control oils. The cholesterol/phospholipid molar ratio in the liver microsomal fraction was about 0.26. This is in agreement with the values found in the endoplasmic reticulum of rats (21). The phospholipid composition is also given in Table 1. It can be seen that the percentages of the major phospholipids were not affected by the dietary oils, consistent with other reports (22,23). The major phospholipid classes were phosphatidylcholine (50–53%), phosphatidylethanolamine (28–32%), and phosphatidylserine plus phosphatidylinositol (9–13%), representing 92% of the total. Other phospholipids were present at less than 3.5%. This profile is in accordance with that described by Diagne *et al.* (24) in guinea pig liver.

Table 2 shows the relative fatty acid composition of liver microsomal lipids in the control guinea pigs (fed no oil) and in the guinea pigs fed test oils. Linoleic (18:2n-6), palmitic (16:0), stearic (18:0), and oleic (18:1n-9) acids were the predominant fatty acids in all groups, representing about 91% of the total. Other fatty acids were present at percentages below 3.5%.

Microsomal lipids from guinea pigs fed a standard diet supplemented with nonheated control oil showed several changes in fatty acid composition, the most striking being a significant increase in the relative stearic (55.5%), linoleic (21%), and arachidonic (117%) acid levels, with a concomitant decrease in the percentages of palmitic (43%), palmitoleic (16:1n-7, 65%), oleic (39%), and α-linolenic (70%) acids. These results are in general agreement with those of other studies in which increased levels of n-6 polyunsaturated fatty acids were found in the tissues of animals fed corn (9,10,12), soybean (11), and sunflower (11,25–28) oils. Diets rich in linoleic acid typically result in its large uptake and an increase in the contents of n-6 fatty acid in phospholipids from different tissues.

The fatty acid profile from the animals fed nonheated case oil displayed a behavior similar to that observed in the animals fed with nonheated control oil. Thus, the changes should be attributed exclusively to the dietary oil.

Ingestion of heated control oils, which have the lowest content of linoleic acid, induced changes in the fatty acid composition of microsomal lipids similar to those induced by nonheated control oil diets. However, the 18:1/18:0 ratio reached values higher than those obtained in animals fed nonheated oil. Similarly, the administration of heated case oil produced the highest increase in the 18:1/18:0 ratio. These results are in a good agreement with those found in rat liver microsomes from animals fed heated oils (29).

In conclusion, our data demonstrate that the administration of dietary case oil produces changes in the liver microsomal lipid composition, which may be attributable to the pres-

TABLE 2
Comparative Studies of Fatty Acid Composition of Lipid Microsomes from Liver Guinea Pigs After Administration of Control and Case Oils[a]

Major fatty acids	Control (no oil)	Control oil		Case oil	
		Nonheated	Heated	Nonheated	Heated
14:0	1.4 ± 0.2[b]	0.2 ± 0.0[c]	0.4 ± 0.1[c]	0.5 ± 0.2	0.4 ± 0.1
16:0	26.7 ± 0.8[b]	15.1 ± 0.6[c]	17.4 ± 1.0[c]	19.2 ± 0.7	17.4 ± 0.5
16:1n-7	2.0 ± 0.2[b]	0.7 ± 0.1[c]	1.1 ± 0.1[c]	1.1 ± 0.1	1.3 ± 0.1
17:0	0.7 ± 0.0[b,c]	0.9 ± 0.1[b,d]	0.5 ± 0.0[c,e]	0.8 ± 0.0[d,e]	0.6 ± 0.0[e]
18:0	18.0 ± 1.1[b]	28.0 ± 0.7[c,d]	21.4 ± 0.8[b,e]	25.4 ± 0.5[d,e]	19.8 ± 1.6[e]
cis 18:1n-9	15.6 ± 0.7[b]	9.5 ± 0.6[c,d]	14.0 ± 1.0[b–e]	11.9 ± 0.3[d]	17.8 ± 1.3[e]
18:2n-6	29.5 ± 0.4[b]	35.6 ± 0.7[c]	34.8 ± 0.9[b–e]	33.5 ± 0.5	32.2 ± 0.7
18:3n-3	3.0 ± 0.2[b]	0.9 ± 0.1[c,d]	2.1 ± 0.1[b–e]	1.2 ± 0.1[d]	3.3 ± 0.3[e]
20:2n-9	0.9 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	1.0 ± 0.6	0.7 ± 0.2
20:4n-6	2.3 ± 0.1[b]	5.0 ± 0.4[c]	5.6 ± 0.5[c]	3.8 ± 0.2	3.9 ± 0.3
SFA	46.0 ± 1.1	45.1 ± 1.3[d]	40.8 ± 1.0[d,e]	46.3 ± 0.9[d]	38.0 ± 0.3[e]
MUFA	18.1 ± 0.9[b]	10.5 ± 0.8[c,d]	15.2 ± 1.2[b–e]	12.6 ± 0.5[d]	19.3 ± 1.4[e]
PUFA	35.8 ± 0.5[b]	43.1 ± 1.2[c]	42.7 ± 1.4[b,c]	39.4 ± 0.9	40.7 ± 0.9
UFA	54.0 ± 1.1	53.5 ± 1.3[d]	56.9 ± 1.2[d,e]	52.1 ± 0.6[d]	61.2 ± 0.4[e]
SFA/UFA	0.86 ± 0.04	0.85 ± 0.04[d]	0.71 ± 0.03[d,e]	0.89 ± 0.03[d]	0.62 ± 0.01[e]
Unsaturation index[f]	97.6 ± 1.6	109.8 ± 4.0	112.8 ± 3.2	100.3 ± 2.0	111.6 ± 2.7

[a]Values are means ± SE for eight animals per dietary group. Fatty acids contributing less than 0.5% are omitted: 15:0, 18:4, 19:0, 20:0, 20:1, 21:0, 22:5, and 22:6 + 24:0. Unknown fatty acid methyl esters represent about 0.5–0.7% of the total. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UFA, unsaturated fatty acids.

[b,c]By rows, values with different superscripts are significantly different at $P < 0.01$, for comparisons between control and control oil (diet effects).

[d,e]By rows, values with different superscripts are significantly different at $P < 0.01$, for comparisons between control oil and case oil (case oil effects).

[f]The unsaturation index was calculated as the sum of percentages by weight of each fatty acid times the number of olefinic bonds.

ence of aniline and fatty acid anilides in the samples. The failure to detect additional effects could be due to an insufficient quantity of TOS agent administered, as suggested by Kilbourne *et al.* (6). However, it could also be due to a clearance of the TOS agent by detoxifying enzyme systems, because it is well known that dietary and nutritional factors are able to modulate such enzyme systems (30). In fact, there is evidence that diets containing high levels of linoleic acid, such as corn oil, increase the mixed function oxidase activity and cytochrome P_{450} in rat liver (31).

ACKNOWLEDGMENTS

This work was supported by grant 88/2572, from the Fondo de Investigación Sanitaria, Ministerio de Sanidad y Consumo, and by grant SA-56/10/92 from Consejeria de Cultura y Turismo, Junta de Castilla y León. The authors are grateful for proofreading by N.S.D. Skinner.

REFERENCES

1. Kilbourne, E.M., Rigau Pérez, J.G., Heath Jr., C.W., Zack, M.M., Falk, H., Martín-Maros, M., and DeCarlos, A. (1983) Clinical Epidemiology of Toxic-Oil Syndrome. Manifestations of a New Illness, *New Engl. J. Med. 309*, 1408–1414.
2. Tabuenca, J.M. (1981) Toxic-Allergic Syndrome Caused by Ingestion of Rapeseed Oil Denatured with Aniline, *Lancet 2*, 567–568.
3. Pestaña, A., and Muñoz, E. (1982) Anilides and the Spanish Toxic Oil Syndrome, *Nature 298*, 608.
4. Aldridge, W.N. (1992) The Toxic Oil Syndrome (TOS, 1981): From the Disease Towards a Toxicological Understanding of Its Chemical Aetiology and Mechanism, *Toxicol. Lett. 64/65*, 59–70.
5. Posada de la Paz, M., Philen, R.M., Abaitua Borda, I., Bernert Jr., J.T., Beda Gancedo, J.C., Du Clos, P.J., and Kilbourne, E.M. (1991) Manufacturing Processes at Two French Rapeseed Oil Companies: Possible Relationships to Toxic Oil Syndrome in Spain, *Food Chem. Toxicol. 29*, 797–803.
6. Kilbourne, E.M., Bernert Jr., J.T., Posada de la Paz, M., Hill Jr., R.H., Abaitua Borda, I., Kilbourne, B.W., Zack, M.M., and The Toxico-Epidemiologic Group: Perez, J.A.C., de Rojas, F.D., Pinilla, R.F., Montes, P.G., de la Fuente, G., Benito, J.N.L.S., Benito, O., Tabuenca, J.M., Falk, H., Hannon, W.H., Needham, L.L., Sampson, E., and Turner, W.E. (1988) Chemical Correlates of Pathogenicity of Oils Related to the Toxic Oil Syndrome Epidemic in Spain, *Am. J. Epidemiol. 127*, 1210–1227.
7. Velicia, R., Sanz, C., Martínez-Barredo, F., Sanchez-Tapias, J.M., Bruguera, M., and Rodés, J. (1986) Hepatic Disease in the Spanish Toxic Oil Syndrome. A Thirty Months Follow-Up Study, *J. Hepatol. 3*, 59–65.
8. Solís Herruzo, J.A., Vidal, J.V., Colina, F., Santalla, F., and Castellano, G., (1986) Nodular Regenerative Hyperplasia of the Liver Associated with the Toxic Oil Syndrome: Report of Five Cases, *Hepatology 6*, 687–693.
9. Hammer, C.T., and Wills, E.D. (1978) The Role of Lipid Components of the Diet in the Regulation of the Fatty Acid Composition of the Rat Liver Endoplasmic Reticulum and Lipid Peroxidation, *Biochem. J. 174*, 585–593.
10. Hammer, C.T., and Wills, E.D. (1979) The Effect of Dietary Fats on the Composition of the Liver Endoplasmic Reticulum and Oxidative Drug Metabolism, *Br. J. Nutr. 41*, 465–475.
11. Tahin, Q.S., Blum, M., and Carafoli, E. (1981) The Fatty Acid Composition of Subcellular Membranes of Rat Liver, Heart, and Brain: Diet-Induced Modifications, *Eur. J. Biochem. 121*, 5–13.
12. Periago, J.L., Pita, M.L., Sánchez del Castillo, M.A., Caamaño, G., and Suárez, M.D. (1989) Changes in Lipid Composition of Liver Microsomes and Fatty Acyl-CoA Desaturase Activities Induced by Medium Chain Triglyceride Feeding, *Lipids 24*, 383–388.
13. McMurchie, E.J., Gibson, R.A., Charnock, J.S., and McIntosch, G.H. (1986) Mitochondrial Membrane Fatty Acid Composition in the Marmoset Monkey Following Dietary Lipid Supplementation, *Lipids 21*, 315–323.
14. Lake, B.G. (1987) Preparation and Characterisation of Microsomal Fractions for Studies on Xenobiotic Metabolism, in *Biochemical Toxicology. A Practical Approach* (Snell, K., and Mulloch, B., eds.) pp. 183–215, IRL Press, Oxford.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
16. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37*, 911–915.
17. Kates, M. (1972) General Analytical Procedures, in *Laboratory Techniques in Biochemistry and Molecular Biology. Techniques of Lipidology* (Work, T.S., and Work, E., eds.) 1st edn., pp. 360–361, North-Holland American, Elsevier.
18. Rouser, G., Fleischer, S., and Yamamoto, V. (1970) Two-Dimensional Thin-Layer Chromatographic Separation of Polar Lipids and Determination of Phospholipids by Phosphorus Analysis of Spots, *Lipids 5*, 494–496.
19. Sánchez-Yagüe, J., and Llanillo, M. (1986) Lipid Composition of Subcellular Particles from Sheep Platelets. Location of Phosphatidylethanolamine and Phosphatidylserine in Plasma Membranes and Platelet Liposomes, *Biochim. Biophys. Acta. 856*, 193–201.
20. Metcalfe, L.V., Schmitz, A.A., and Pelke, J.R. (1966) Rapid Preparation of Fatty Esters from Lipids for Gas Chromatographic Analysis, *Anal. Chem. 38*, 514–516.
21. Dawidowicz, E.A. (1987) Dynamics of Membrane Lipid Metabolism and Turnover, *Ann. Rev. Biochem. 56*, 43–61.
22. Lèger, C.L., Christon, R., Viret, J., Daveloose, D., Mitjavila, S., and Even, V. (1989) Nutrition and Biomembranes: Additional Information Concerning the Incidence of Dietary Polyunsaturated Fatty Acids on Membrane Organization and Biological Activity, *Biochimie 71*, 159–165.
23. Garg, M.L., Sebokova, E., Thompson, A.B.R., and Clandinin, M.T. (1988) Delta 6-Desaturase Activity in Liver Microsomes of Rats Fed Diets Enriched with Cholesterol and/or Omega 3 Fatty Acids, *Biochem. J. 249*, 351–356.
24. Diagne, A., Fauvel, J., Record, M., Chap, H., and Douste-Blazy, L. (1984) Studies on Ether Phospholipids. II. Comparative Composition of Various Tissues from Human, Rat, and Guinea Pig, *Biochim. Biophys. Acta. 793*, 221–231.
25. Girón, M.D., Mataix, F.J., Faus, M.J., and Suárez, M.D. (1989) Effect of Long-Term Feeding Olive and Sunflower Oils on Fatty Acid Composition and Desaturation Activities of Liver Microsomes, *Biochem. Int. 19*, 645–656.
26. Girón, M.D., Mataix, F.J., and Suárez, M.D. (1990) Changes in Lipid Composition and Desaturase Activities of Duodenal Mucosa Induced by Dietary Fat, *Biochim. Biophys. Acta. 1045*, 69–73.
27. Navarro, M.D., Hortelano, P., Periago, J.L., and Pita, M.L. (1992) Effect of Dietary Olive and Sunflower Oils on the Lipid Composition of the Aorta and Platelets and on Blood Eicosanoids in Rats, *Atherosclerosis and Thrombosis 12*, 830–835.

28. Muci, M.R., Capello, A.R., Vonghia, G., Belleti, E., Zezza, L., and Guoni, G.V. (1992) Change in Cholesterol Levels and in Lipid Fatty Acid Composition in Safflower Oil Fed Lambs, *Internat. J. Vit. Nutr. Res. 62*, 330–333.
29. Ruiz-Gutiérrez, V., and Muriana, F.J.G. (1992) Effect of Ingestion of Thermally Oxidized Frying Oil on Desaturase Activities and Fluidity in Rat Liver Microsomes, *J. Nutr. Biochem. 3*, 75–79.
30. Yoo, J-S. H., Smith, T.J., Ning, S.M., Lee, M-J., Thomas, P.E., and Yang, C.S. (1992) Modulation of the Levels of Cytochromes P450 in Rat Liver and Lung by Dietary Lipid, *Biochem. Pharmacol. 43*, 2535–2542.
31. Roowe, L., and Willis, E.D. (1976) The Effect of Dietary Lipids and Vitamin E on Lipid Peroxide Formation, Cytochrome P-450 and Oxidative Demethylation in the Endoplasmic Reticulum, *Biochem. Pharmacol. 25*, 175–179.

[Received May 7, 1994, and in final revised form March 17, 1995; Revision accepted April 4, 1995]

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) Temperature and Diet Effects on Trout, *J. Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) Temperature and Diet Effects on Trout, in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., Temperature and Diet Effects on Trout, *J. Biol. Chem.*, in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

AVAILABILITY OF FISH OIL TEST MATERIALS

The Fish Oil Test Materials Program is a cooperative effort of the National Institutes of Health (NIH) and the National Oceanic and Atmospheric Administration/Department of Commerce (NOAA/DOC). This program provides a long-term, consistent supply of quality-assured/quality-controlled test materials to researchers that facilitates the evaluation of ω-3 fatty acids in health and disease.

Current test materials available, in both capsules and bulk, include: (i) vacuum deodorized menhaden oil; (ii) ethyl esters of olive, corn, and safflower oils; (iii) commercial preparations of corn, olive, and safflower oil; (iv) n-3 ethyl ester concentrates; (v) EPA ethyl ester; and (vi) DHA ethyl ester.

To qualify to receive the fish oil test materials from the Program, the researcher must fill out an application. The applicant must: (i) have peer-reviewed research indicating the need for the requested materials, and (ii) submit a cor-

(continued on next page)

rectly completed application form and a signed waiver of liability. *The awarded materials are provided free of charge (researchers will pay shipping costs).*

Investigators may obtain further information and an application form from Fish Oil Test Materials Program, NIAAA, National Institutes of Health, 12501 Washington Ave., Flow Building, Room 2, Rockville, MD 20852 (Phone: Beth Aurecchia, 301-443-9643; Fax: 301-594-0035).

CALENDAR

August 1995

36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September 1995

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October 1995

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

American College of Nutrition 36th Annual Meeting, Oct. 13–15, 1995, Omni Shoreham Hotel, Washington, D.C. Information: American College of Nutrition, c/o Hospital for Joint Disease, 301 E. 17th St., New York, NY 10003 (Fax: 212-777-1103).

Lipidforum Seminar: Lipids in Animal Nutrition, Oct. 25–26, 1995, Research Centre Foulum, Tjele (Viborg), Denmark. Information: G. Lambertsen, Lipidforum, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-32 57 24).

November 1995

XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

February 1996

Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

Structure and Biosynthesis of Novel Conjugated Polyene Fatty Acids from the Marine Green Alga *Anadyomene stellata*

Marina V. Mikhailova[a], Debra L. Bemis[a], Mitchell L. Wise[b], William H. Gerwick[b], James N. Norris[c] and Robert S. Jacobs[a,*]

[a]Marine Science Institute, University of California, Santa Barbara, California 93106, [b]College of Pharmacy and Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, and [c]Department of Botany, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560

ABSTRACT: Novel polyunsaturated fatty acids with four conjugated double bonds were found in extracts of the green macroalga, *Anadyomene stellata*. The isolation of five of these with different chain lengths and varying degrees of unsaturation—16:5, 18:4, 20:5, 20:6, and 22:7—was accomplished by organic extraction followed by a combination of vacuum and high-performance liquid chromatography. One of these that was a novel substance (22:7) was characterized as 4*Z*,7*Z*,9*E*,11*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid and assigned the trivial name stellaheptaenoic acid. The structure of this new compound, isolated as its methyl ester derivative, was deduced from detailed nuclear magnetic resonance, gas chromatography/mass spectrometry (GC/MS), and other spectroscopic methods. Incubation of a chloroplast preparation, isolated from a crude algal homogenate by differential centrifugation, with six unsaturated fatty acids (palmitoleic, 6*Z*,9*Z*,12*Z*,15*Z*-octadecatetraenoic acid, arachidonic acid, eicosapentaenoic acid, 7*Z*,10*Z*,13*Z*,16*Z*-docosatetraenoic acid, and 4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid) resulted in substantially increased synthesis of unique tetraene compounds as detected by ultraviolet spectrophotometry and tentatively identified by GC/MS.

Lipids 30, 583–589 (1995).

Despite the many differences between marine and terrestrial organisms at the cellular and biochemical level, the application of a comparative approach has proven to be successful in the elucidation of many shared physiological and biochemical processes in these classes of life forms (1). The oxidation of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), to biologically active compounds is one such process. The capacity to utilize this fatty acid and produce metabolites with structures similar to prostaglandins and leukotrienes, mediators of inflammation among other functions, has been found in such marine organisms as coralline red algae (Corallinales) and tunicates (Urochordata) (2–6). The biological functions of these enzymatic cascades and resulting metabolites, along with the evolutionary implications of such similarities, are the focus of our interests.

Additionally, compounds derived from some sponges (Porifera) and soft corals (Anthozoa: Gorgonacea) have been found to inhibit the signaling cascade involved in inflammation in mammalian models. A few examples include fuscoside, scalaradial, and manoalide, which were isolated from the gorgonian, *Eunicea fusca*, a sponge from the genus *Cacospongia*, and another sponge, *Luffariella variabilis*, respectively (7–12). Thus, not only do marine invertebrates and algae provide us with unique models to study fatty acid metabolism and other biochemical pathways, but they provide us with new molecular probes with which to study the process of inflammation in mammalian systems.

Oxylipins (13), oxidative metabolites of AA and its homologues, are found in four kingdoms, Eubacteria, Protista, Plantae, and Animalia, suggesting that they possess important physiological roles. Among the marine algal macrophytes, various chain length PUFAs and resulting oxylipins have been found in all of the major multicellular groups (Rhodophyta, Phaeophyceae, and Chlorophyta) (4,6). Cyanobacteria, on the other hand, appear to metabolize shorter chain fatty acids of 14 to 18 carbon atoms in length (4,6,14).

The ability of marine algae to produce PUFAs, particularly 20- and 22-carbon ω3 fatty acids, has long been recognized (15). In addition, enzyme(s) have been found that are capable of producing eicosanoids *via* oxidation of 20-carbon PUFAs. The biochemical pathways involved have been considered to some extent in marine red algae (4,6); however, little work has been done with marine green algae. Furthermore, the physiological significance of the metabolites produced in these groups of algae, as well as many other organisms, is largely

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; BPA, bosseopentaenoic acid; COSY, ^{1}H-^{1}H chemical shift correlation spectroscopy; DTT, DL-dithiothreitol; EPA, eicosapentaenoic acid; EtOAc, ethyl acetate; FAMEs, fatty acid methyl esters; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; PMSF, phenylmethylsulfonyl fluoride; PUFA, polyunsaturated fatty acid; PVPP, polyvinylpolypyrrolidone; SHA, stellaheptaenoic acid; TLC, thin-layer chromatography; UV, ultraviolet.

Copyright © 1995 by AOCS Press

unknown. It has been a frequent observation that some marine algae possess enzyme systems capable of metabolizing 20-carbon PUFAs as well as 18- and 22-carbon PUFAs, resulting in the production of diverse chain length oxylipins. However, in most cases the substrate preference and specific functions of the enzyme cascade(s) involved are still to be determined.

In 1991 we undertook a study of selected red and green marine algae to determine the relative capacity of various organisms to metabolize AA (Bober, M.A., Norris, J.N., Williams, A.B., and Jacobs, R.S., submitted for publication). Among the 27 benthic marine algal species collected off the central Atlantic coast of Florida and in the Florida Keys, we detected high levels of AA metabolic activity in a green algal species, *Anadyomene stellata.* This alga is frequently found in shallow marine habitats exposed to high herbivory, yet does not appear to be preyed upon. This observation has lead to suggestions that it is a fairly unpalatable species among the family Anadyomenaceae (16,17).

Several characteristics of *A. stellata* made it an attractive species in which to study PUFA metabolism, the biological roles of its metabolites, and their possible pharmaceutical applications. These include the relative ease of collection of this alga, and though patchy in distribution, it is often abundant where found. This species is found from low intertidal to the shallow subtidal regions in subtropical to tropical waters of the Western Atlantic and Mediterranean (18). *Anadyomene stellata* is often found growing sympatrically with *A. saldanhae*. It is distinguished from the latter by the characteristics of the branching pattern of the veins and cells of their unistratose blades, as seen under a dissection microscope. Furthermore, the uniserate veins and cells between the veins are large and easily visible under a light microscope, rendering them accessible for observation and experimentation both *in vivo* and *in vitro.*

MATERIALS AND METHODS

Algae. Anadyomene stellata (Wulfen) C. Agardh (Anadyomenaceae, Cladophorales) was collected in 0.3 to 2.0 meters of water off the north coast of Long Key, Florida (April and September 1994), cleaned of infauna, epizooa, and epiphytes, damp dried, immediately frozen in liquid nitrogen, and transported to California on dry ice, and stored at –77°C. Taxonomic voucher specimens of the species studied are deposited in the Algal Collection, U.S. National Herbarium, National Museum of Natural History, Smithsonian Institution (Washington, D.C.). Field work was conducted at the Keys Marine Laboratory, Long Key, and laboratory work at the Smithsonian Marine Station at Link Port (Fort Pierce, FL) and the University of California (Santa Barbara, CA).

Materials. Polyvinylpolypyrrolidone (PVPP), phenylmethylsulfonyl fluoride (PMSF), DL-dithiothreitol (DTT), EDTA, and deuterated benzene were obtained from Sigma Chemical Co. (St. Louis, MO). Palmitoleic, 6Z,9Z,12Z,15Z-octadecatetraenoic acid, AA, eicosapentaenoic acid (EPA), 7Z,10Z,13Z,16Z-docosatetraenoic acid, and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid were also obtained from Sigma Chemical Co. High-performance liquid chromatography (HPLC) grade organic solvents and thin-layer chromatography (TLC) grade silica gel were obtained from Fisher Scientific (Pittsburgh, PA) or by fresh distillation. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) was obtained from Aldrich Chemical Co. (Milwaukee, WI), and Miracloth from Calbiochem (La Jolla, CA).

Lipid preparation. Portions of approximately 100 g of frozen algal thalli were ground in liquid nitrogen. The coarse powder was added to chloroform/methanol (2:1) and homogenized at 0°C with a Tekmar (Cincinnati, OH) homogenizer. The homogenate was then filtered through a Buchner funnel fitted with Whatman (Clifton, NJ) filter paper #1. For further purification, distilled water was added to the mixture along with 1 M NaCl in a 1:10 final volume. The water phase was discarded, and the organic phase dried with anhydrous Na_2SO_4, filtered, and reduced *in vacuo*. The dry sample was resuspended in 1 mL hexane and methylated with diazomethane (19).

Preparation of chloroplast fraction. Chloroplasts were obtained from 25 g of frozen algal specimens by the following procedure. Thalli of thawed algae were first ground with a mortar and pestle using a small amount of purified silica to aid in the grinding process and then suspended in 40 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing 5 mM EDTA, 5 mM DTT, and 1 mM PMSF. The mixture was filtered through one layer of Miracloth and centrifuged for 1 min at 400 × *g* (Beckman TJ-6; Beckman, Fullerton, CA) with 3% wt/vol PVPP. The supernatant was filtered through a layer of Miracloth and centrifuged for 5 min at 1000 × *g*. This chloroplast-enriched pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, to a concentration of 60 µg/mL as determined by the methods of Bradford (20), and then used for incubations with fatty acid substrates.

Incubation with fatty acid substrates and extraction of products. Six unsaturated fatty acids not containing conjugated double bonds (palmitoleic, 6Z,9Z,12Z,15Z-octadecatetraenoic acid, AA, EPA, 7Z,10Z,13Z,16Z-docosatetraenoic acid, and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid) were used as substrates for incubation with the chloroplast fraction. Incubations were prepared in triplicate by adding a fatty acid substrate dissolved in EtOH to a 500 µL aliquot of the chloroplast fraction (200 µM final concentration). The incubations were mixed continuously for 3 h at room temperature (*ca.* 22°C). Following the incubation period, the lipids were extracted in 3 mL of hexane, and dried to completion under nitrogen. Ultraviolet (UV) spectrophotometric analysis of the samples was conducted following reconstitution in a small volume of hexane. Controls, with no substrate added, were treated as described above.

Chromatographic and instrumental methods. Methyl esters were chromatographed over TLC grade silica gel under vacuum with increasing percentages of ethyl acetate (EtOAc) in hexane. After separation, the fractions were scanned in the UV range with an IBM (Danbury, CT) 9430 UV-VIS scan-

ning spectrophotometer or on a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer. TLC of 1 μL of this extract (0.2% of total) was conducted on Whatman TLC plates (silica gel 60 A) in a solvent system of either hexane/diethyl ether/acetic acid (85:15:1, by vol) or EtOAc/hexane (2:98, vol/vol). Further purification of methyl esters was achieved by both reverse- and straight-phase HPLC using either a Hitachi (Mountain View, CA) 4200 instrument or a Waters (Milford, MA) M-6000A pump with a Waters Lambda Max 480 UV detector. Reverse-phase HPLC was performed over Partisil 5 ODS-3 (250 × 4.6 mm) (Whatman) with a gradient of acetonitrile/tetrahydrofuran/water (9:5:11) (15) (solvent A) and methanol (solvent B) as follows: 0 min 100% A; 10 min 99% A and 1% B; 20 min 95% A and 5% B; 45 min 90% A and 10% B. Detection was at 328 nm with a flow rate of 3 mL/min. Straight-phase HPLC was conducted on two 4 mm × 30 cm Alltech (Deerfield, IL) 10 μ silica columns connected in series. An isocratic solvent system of EtOAc/hexane (1:99, vol/vol) was used with a 3 mL/min flow rate and 328 nm detection.

Gas chromatography/mass spectrometry (GC/MS) of derived natural products from *A. stellata* was performed with a Hewlett-Packard Model 5890 Series II gas chromatograph and an HP5971A quadrapole mass selective detector interfaced with a Hewlett-Packard Chemstation using G1034B software for data analysis. An 11.5 meter Ultra 1 (100% dimethylpolysiloxane) capillary column was used in the splitless injection mode. Injector temperature was 250°C, and oven temperature was 100°C with a 10°C/min temperature ramp to a final temperature 240°C (detector temperature 280°C). Helium was used as the carrier at 11 psi. GC/MS of samples from incubation experiments was performed on a modified HP 5985B GC/MS instrument. Samples were loaded onto a solvent-free GC injector (dropping needle type; Ray Allen Associates, Boulder, CO) connected to a bonded phase fused silica capillary column (DB-1, 15 m, 0.26 mm i.d., film thickness 0.25 μM; J&W Scientific, Folsom, CA). Injector temperature was 250°C, and oven temperature was set at 80°C for 1 min with a 7°C/min temperature ramp up to a final temperature of 300°C. Helium was used as the carrier gas. Nuclear magnetic resonance (NMR) experiments were conducted on either Bruker ACP 300 MHz or AM 400 MHz instruments.

RESULTS AND DISCUSSION

Isolation of tetraene compounds from A. stellata. The UV-spectrum of the lipid fraction of the algae in hexane with absorption maxima at 294, 306, and 322 nm (Fig. 1) indicates the presence of conjugated tetraene structures (21). However, preliminary observations of the crude extract by UV spectrophotometry suggested that these compounds were chemically unstable. Hence, the crude lipid extract was methylated with diazomethane which improved the stability and chromatographic characteristics of these compounds. These derivatives were stable at −77°C under nitrogen for one month with no detectable decomposition by TLC. For further purification, the methyl esters were separated by vacuum liquid chromatography using mixtures of EtOAc and hexane from 0 to 100% EtOAc. With the use of UV spectrophotometry, compounds with tetraene structures were detected in the third fraction eluting with EtOAc/hexane (1:99, vol/vol). This fraction was dried and its components separated with straight-phase HPLC. Compounds showing significant adsorption at 328 nm eluted in three fractions centered at 42, 48, and 54 mL. These fractions were collected, reduced *in vacuo*, and analyzed by GC/MS and NMR.

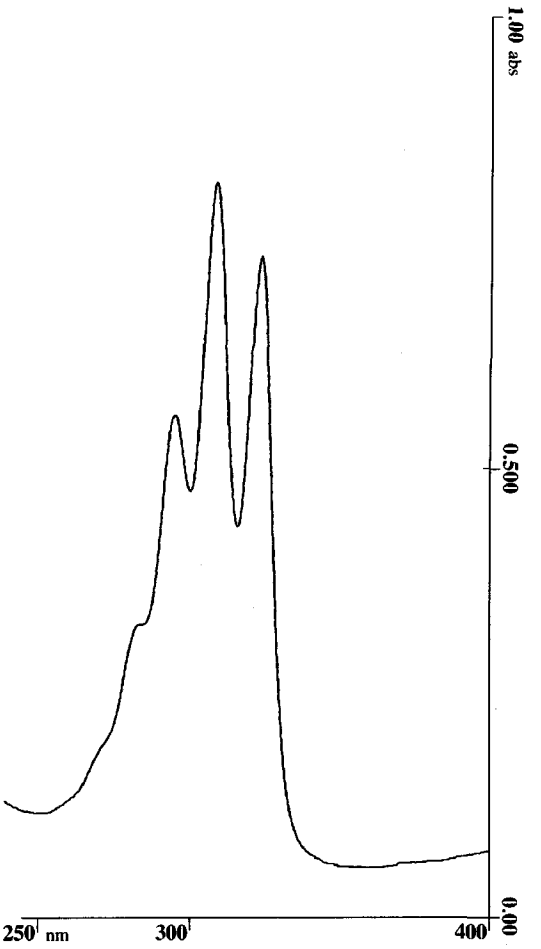


FIG. 1. Ultraviolet spectrophotometric scan of organic extract of *Anadyomene stellata*.

GC/MS and NMR analyses of methylated tetraenes. The three HPLC fractions (Fig. 2) showing significant UV absorption at 328 nm all had UV spectra with maxima at 294, 306, and 322 nm, characteristic of conjugated tetraenes. The fraction eluting at 54 mL showed (Fig. 3) a single major peak by GC/MS, derivative **2**, with an $M^+ = 340$, corresponding to a molecular formula of $C_{23}H_{32}0_2$ [LR EIMS *m/z* (rel. intensity) obs.[M^+] *m/z* 340 (6), 260 (18), 232 (30), 171 (10), 157 (21), 145 (27), 131 (83), 117 (83), 105 (63), 91 (100), 79 (57), 67 (30)]. Assuming an $\varepsilon \cong 72{,}000\ M^{-1}cm^{-1}$ (9) for the UV maxima at 306 nm, 0.53 mg of derivative **2** was isolated in this process (Scheme 1—structural formulas for conjugated tetraene metabolites and derivatives discussed in text). The



FIG. 2. High-performance liquid chromatography-straight phase chromatogram of fraction 3 eluted in 1% ethyl acetate in hexane by vacuum liquid chromatography: peaks 1 and 3—mixture of methyl ester derivatives having molecular ions *m/z* 260, 290, 314, 316; peak 2—methyl bosseopentaenoate, molecular ion *m/z* 316; and peak 4—methyl ester derivative having molecular ion *m/z* 340.

infrared spectrum for **2** showed the following: (neat) 3015, 2959, 2926, 2874, 1740, 1717, 1457, 1436, 1251, 1196, 1163, 995, 955, 668 cm^{-1}. The ^1H NMR (300 MHz) in d_6-benzene showed the following (shifts relative to solvent peak at 7.15 ppm): δ 0.90 (3H, *t*, *J* = 7.51 Hz, H-22), 1.99 (2H, *m*, H-21), 2.11 (2H, *t*, *J* = 7.45 Hz, H-2), 2.30 (2H, *dt*, *J* = 7.3,7.3 Hz, H-3), 2.81 (2H, *dd*, *J* = 5.0, 5.0 Hz, H-18), 2.92 (4H, *m*, H-6,15), 3.32 (3H, *s*, OMe), 5.31–5.44, (8H, *m*, H-4,5,7,14,16,17,19,20), 6.10 (2H, *m*, H-8,13), 6.24 (2H, *m*,

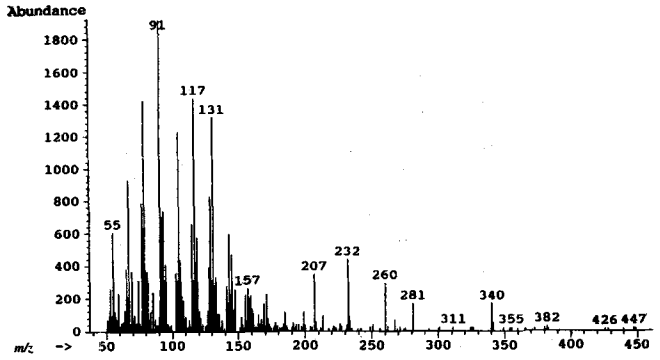
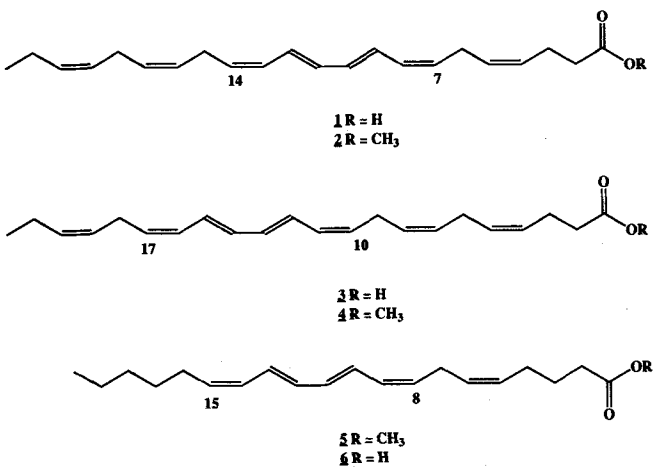


FIG. 3. Electron impact mass spectrum of methyl 4*Z*,7*Z*,9*E*,11*E*,-13*Z*,16*Z*,19*Z*-docosaheptaenoate (**2**).



SCHEME 1

H-10,11), 6.55 (2H, *m*, H-9,12). A ^{13}C distortionless enhancement by polarization transfer experiment (100 MHz) showed the following [shifts relative to solvent at 128.00 ppm, assignments based on model compounds (1,2)]: δ14.44 (C-22; CH_3), 20.89 (C-21; CH_2), 23.09 (C-3; CH_2), 25.92 (CH_2), 26.51 (CH_2), 26.61 (C-18,15,6; CH_2's), 33.94 (C-2, CH_2), 51.98 ($-OCH_3$), 127.33 (C-9; CH), 128.49, (C-8; CH), 128.52 (C-12; CH), 128.54 (C-4; CH), 128.69 (C-7; CH), 128.98 (C-5; CH), 129.19 (C-13; CH), 129.50 (C-19; CH), 129.51 (C-17; CH), 130.37 (C-16;CH), 130.41 (C-20, CH), 132.18 (C-14; CH), 133.84 (C-10; CH), 133.86 (C-11; CH). Resolution of the low field signals was accomplished by Guassian multiplication of the DEPT spectrum from 126 to 136 ppm. A heteronuclear multiple bond coherence experiment showed long range coupling from H_2-2 and the OMe protons to a quaternary carbon (carbonyl) at approximately δ173 ppm and also confirmed assignments of C-3 and C-21.

From these data, it was clear that there were fourteen olefinic methines, six aliphatic methylenes, two methyl groups, and one carbonyl carbon. Three of the methylenes were bisallylic groups as indicated by their chemical shifts, one at δ2.81 and two at δ2.92. A ^1H-^1H chemical shift correlation spectroscopy (COSY) experiment demonstrated coupling from the C2-methylene at δ2.11 through the aliphatic protons at δ2.30 ppm to the C4 olefinic proton at approximately δ5.3–5.4. Similarly, coupling from the terminal methyl at δ0.90 through the methylene protons at δ1.99 to the cluster of olefinic protons at δ5.3–5.4 established that portion of the molecule. The relative positions in the conjugated tetraene functionality of the six low-field proton signals at δ6.10, δ6.24, and δ6.55 (2H each) were assigned by comparison with methyl bosseopentaenoate **5** (22), and were consistent with the COSY data obtained for **2** (Scheme 1). The large cluster of signals at δ5.3–5.4 represented the protons in the three isolated olefinic groups as well as the first and last protons in the conjugated system. The ^1H-^1H COSY experiment also showed a long range coupling from the H3 protons to a bisallylic signal at δ2.92 as well as a long-range coupling between the δ2.92 signal and a low-field olefinic proton at

δ6.10. A long-range ^{1}H-^{1}H COSY experiment optimized for 1 Hz convincingly showed coupling from the ω2 protons (δ1.99) to the bisallylic signal at δ2.81. This was confirmed by decoupling the signal at δ2.81 and observing a distinct sharpening of the ω2 signal. These data eliminated the possibility of the conjugated system starting at C4 or C13, leaving only two possible regioisomers for the natural product fatty acid; i.e., 4,7,9,11,13,16,19 docosaheptaenoic acid (**1**), or 4,7,10,12,14,16,19 docosaheptaenoic acid (**3**).

Resolution of these two possibilities was accomplished by two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) in $CDCl_3$. This experiment revealed a distinct nuclear Overhauser effect between the bisallylic proton signals at δ2.82 and δ2.97 (Fig. 4). In this solvent, identification of the δ2.08 signal as H21 and the δ2.82 signal as H18 was accomplished by selectively irradiating the δ2.08 signal and observing a distinct sharpening of the proton signal at δ2.82, resulting from the loss of homoallylic coupling, and the collapse of the methyl triplet (H_3-22 at δ0.98). Having established that the δ2.82 signal corresponded to H18, the nOe results could only be accounted for by structure **2**.

The relative stereochemistry of the seven olefinic groups (4Z,7Z,9E,11E,13Z,16Z,19Z) is based on the high degree of comparability between the low-field region of the ^{1}H NMR spectrum (22), the ^{13}C NMR spectrum (23,24), and the UV/Vis spectrum (25) for this compound with that of methyl bosseapentaenoic acid (BPA) (**5**). Other possible olefin configurations do not seem likely, given the symmetry of the tetraene system as observed by NMR and the precedence of *Z,E,E,Z* configurations in naturally occurring conjugated tetraenes (24,26).

The HPLC fraction eluting at 42 mL also showed only one major peak by GC/MS with a M^+ *m/z* = 316, corresponding to a molecular formula $C_{21}H_{32}O_2$ [LR EIMS *m/z* (rel. intensity) obs. [M^+] *m/z* 316 (56), 245 (9), 201 (14), 147 (11), 145 (18), 143 (13), 131 (52), 117, (73), 105 (52), 91 (100), 79 (44), 67 (30, 55 (13); ^{1}H NMR (300 MHz, d_6-benzene, shifts relative to the solvent peak at 7.15 ppm) δ 0.86 (3H, *t*, *J* = 7.4 Hz, H-20), 1.23 (4H, *m*, H-18,19), 1.31 (2H, *m*, H-17), 1.58 (2H, *tt*, *J* = 7.4,7.4 Hz, H-3), 1.96 (2H, *bdt*, *J* = 7.3, 7.3 Hz, H-4), 2.09 (2H, *t*, *J* = 7.4 Hz, H-2), 2.13 (2H, *dt*, *J* = 7.6, 7.6 Hz, H-16), 2.88 (2H, *bdd*, *J* = 7.4, 7.4 Hz, H-7), 3.32 (3H, *s*, OMe), 5.27 (1H, *dtt*, *J* = 8.3, 8.3, 1.5 Hz, H-5), 5.36–5.47, (3H, *m*, H-6,8,15), 6.09 (1H, *dd*, *J* = 11.0, 11.0 Hz, H-9), 6.14 (1H, *dd*, *J* = 11.0, 11.0 Hz, H-14), 6.25 (1H, *m*, H-12), 6.31 (1H, *m*, H-11), 6.56 (2H, *dd*, *J* = 11.4,13.7 Hz, H-10,13]. [These coupling constants are slightly different than those described in Burgess *et al.* (22). We believe these to be the correct values.] The proton spectrum of this compound was essentially identical to that of BPA (22). However, to confirm its identity as methyl 5*Z*,8*Z*,10*E*,12*E*,14*Z*-eicosapentaenoate (**5**) as opposed to methyl 5*Z*,7*E*,9*E*,11*Z*,14*Z*-eicosapentaenoate, two additional proton decoupling experiments were performed. First, irradiation of the signal at δ1.96 (H_2-4) collapsed the signals at δ1.58 (H_2-3) and δ5.27 (H-5). In turn, irradiation at δ1.58 collapsed the signals at δ1.96 and δ2.09. These results clearly demonstrated that the unsaturation pattern in this isolate of **5** was 5*Z*,8*Z*,10*E*,12*E*,14*Z*, as previously assigned for BPA (**6**).

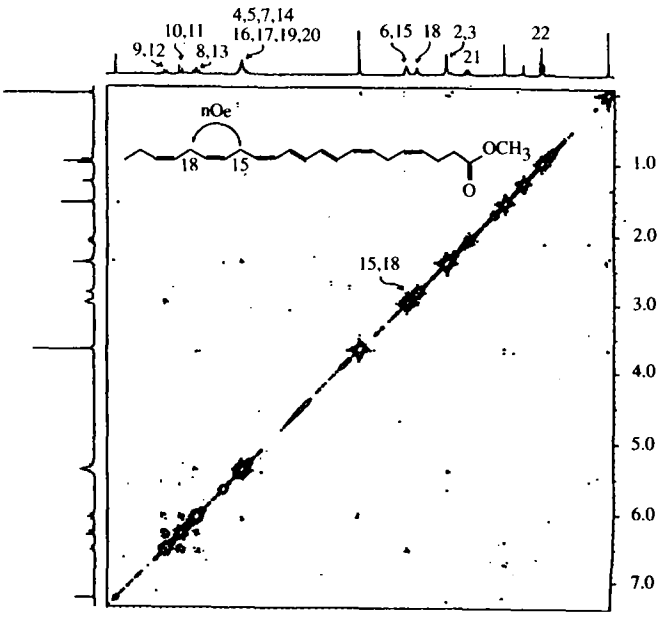
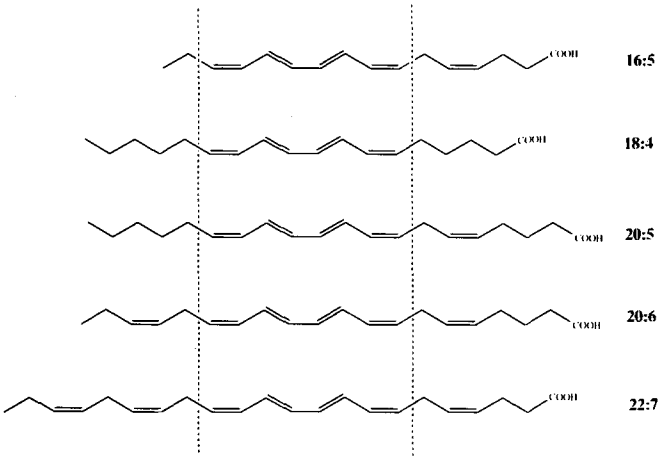


FIG. 4. ^{1}H-^{1}H Nuclear Overhauser enhancement correlation spectroscopy of methyl 4*Z*,7*Z*,9*E*,11*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoate (**2**).

The HPLC peak eluting at 48 mL contained a mixture of four compounds as determined by GC/MS having molecular ions at *m/z* 260, 290, 314, and 316. These ions are consistent with the methyl esters of C16:5 (11.3 min), C18:4 (13.3 min), C20:5 (14.8 min), and C20:6 (15.2 min). Better separation of these fatty acid methyl esters (FAMEs) was achieved by reverse-phase HPLC. Compounds showing significant adsorption at 328 nm eluted in five fractions centered at 118, 192, 197, 222, and 229 mL; these were separated, reduced under vacuum, and analyzed by GC/MS. The fraction eluting at 118 mL showed a single major peak with M^+ 260 corresponding to the methyl ester of 16:5, fraction 192 mL-M^+ 314-C20:6; 197 mL-M^+ 290-C18:4; 222 mL-M^+ 340-C22:7 and 229 mL-M^+ 316-C20:5. While three of these FAMEs, C16:5, C18:4, and C20:6, have not yet been purified to a degree sufficient for rigorous molecular analysis, their structures are tentatively presented here as 16:5Δ4,7,9,11,13, 18:4Δ6,8,10,12, and 20:6Δ5,8,10,12,14,17 (Scheme 2—structures of conjugated tetraene metabolites were aligned so as to show the relationship of the tetraene functionality to the carboxyl terminus. Presumably, this is established as a result of substrate recognition and binding characteristics of the producing enzyme). The structures of the methyl esters of the other two compounds, stellaheptaenoic acid (22:7, 4*Z*,7*Z*,9*E*,11*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoate) and BPA (20:5, 5*Z*,8*Z*,10*E*,12*E*,14*Z*-EPA), were rigorously established (Scheme 1; **2** and **5**).

Analysis of chloroplast incubations by UV spectrophotometry. UV spectrophotometry was utilized to determine the presence of tetraene structures following incubations of



SCHEME 2

chloroplast fractions with PUFA precursors (final concentration of 200 μM). UV scans of the extracts resulting from the incubations were characteristic of tetraene structures with absorption maxima at 294, 306, and 322 nm. Additionally, marked increases in these signals were found in samples incubated with the fatty acid substrates. The largest increases were found in the samples incubated with 6Z,9Z,12Z,15Z-octadecatetraenoic acid which showed a nearly 18-fold increase in absorbance of the 306 nm peak above control. Assays containing AA, EPA, 7Z,10Z,13Z,16Z-docosatetraenoic acid, and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid all had similar levels of tetraene compounds present following incubation. The increase in absorbance of the 306 nm peak for each of these samples was 9.2-, 10.8-, 6.6-, and 8.4-fold above control levels, respectively. The samples incubated with palmitoleic acid showed the smallest increase in absorbance of the 306 nm peak (2.6-fold). These relative differences in absorbance were not quantitated further because precise extinction coefficients are not known in the mixture of products. Samples in which the chloroplast fraction was boiled prior to incubation with AA showed no increase in the magnitude of the tetraene signal, suggesting that tetraene formation is dependent on an enzymatic process (data not shown).

*Analysis of chloroplast incubations by GC/MS.*The fatty acids present in samples incubated with 6,9,12,15-octadecatetraenoic acid were determined by GC/MS of the corresponding methyl ester derivatives to be 16:5, 18:4, 20:5, and 20:6. Which of these represent conjugated tetraenes as unmetabolized PUFAs was not determined by this methodology. In the case of application of AA, only fatty acids with 20 carbons were found, 20:5 and 20:6. In contrast, 16:5, 18:4, 20:5, and 20:6 were detected in the assays with EPA added. Five fatty acids, 16:5, 18:4, 20:5, 20:6, and 22:7, were found in the samples in which 4Z,7Z,10Z,13Z,16Z,19Z-docosahexanoic and 7Z,10Z,13Z,16Z-docosatetraenoic fatty acids were used as substrates. In contrast, 20:6, presumably BPA, was the only fatty acid detected in incubations prepared with exogenous palmitoleic acid.

Our tentative interpretation of these results is that the various exogenously applied fatty acids, or metabolites produced from them, are each eliciting the release of specific profiles of fatty acids from complex lipid species which then potentially feed into this tetraene biosynthetic pathway. These complex lipids may be the galactolipids which are found in abundance in chloroplast membranes. Further work is underway to clarify this apparently complex regulation of the flow of fatty acids into this biochemical pathway, as well as other aspects of the biosynthesis of these novel metabolites.

This study demonstrates the capacity of at least six PUFAs, in either free or complexed form, to serve as substrates for the production of novel tetraene structures *via* an oxidative enzymatic pathway present in *A. stellata.* As suggested by Scheme 2, it appears that the enzyme system introduces the conjugated tetraene at a position recognized in relationship to the carboxyl terminus. Recently, mechanistic features of conjugated tetraene biosynthesis were studied in an unrelated red marine alga, *Lithothamnion corallioides* (Corallinales), using cell free methodology (26). If similar fatty acid oxidase systems are in operation in these taxonomically divergent algal phyla, then this biosynthetic pathway must be of considerable biochemical and physiological significance to have survived evolutionary pressures. We believe *A. stellata* to be a useful model for the analysis of the cellular and molecular mechanisms underlying the biosynthetic pathway of these highly reactive fatty acids.

ACKNOWLEDGMENTS

Work at the University of California, Santa Barbara, was supported in part by the California Sea Grant College Program, grant NA36RGO537 (R.S.J.), and in part by a grant from the Smithsonian Marine Station at Link Port, Florida (J.N.N. and R.S.J.). This study represents SMS contribution number 382. We thank Dr. M. Rice and the staff of the Smithsonian Marine Station for providing important support for research conducted at SMSLP. Also, we acknowledge J. Swanson and W. Gibbs from the Keys Marine Laboratory, Long Key, Florida, for their assistance during the algae collection and for the use of the Keys Marine Laboratory. W. Lee and R. Sims provided important technical assistance during field work. We thank Dr. J. Orjala, College of Pharmacy, Oregon State University, and R. Kohnert, Dept. of Chemistry, Oregon State University, for assistance with collecting NMR data. Work at Oregon State University was supported by the Oregon Sea Grant Program R/BT-1 (W.H.G.).

REFERENCES

1. Meunier, J.C., Sealock, R., Olsen, R., and Changeux, J.P. (1974) Purification and Properties of the Cholinergic Receptor Protein from *Electrophorus electricus* Electric Tissue, *Eur. J. Biochem. 45*, 371–394.
2. Jacobs, R.S., Bober, M.A., Pinto, I., Williams, A.B., Jacobson, P.B., and de Carvalho, M.S. (1993) Pharmacological Studies of Novel Marine Metabolites, in *Advances in Marine Biotechnology: Pharmaceutical and Bioactive Natural Products* (Attaway, D.H., and Zaborsky, O.R., eds.) Vol. 1, pp. 77–99, Plenum Press, New York.
3. Gerwick, W.H., Proteau, P.J., Nagle, D.G., Wise, M.L., Jiang, Z.D., Bernart, M.W., and Hamberg, M. (1993) Biologically Active Oxylipins from Seaweeds, *Hydrobiologia 260/261*, 653–665.
4. Gerwick, W.H., and Bernart, M.W. (1993) Eicosanoids and Related Compounds from Marine Algae, in *Advances in Marine*

Biotechnology: Pharmaceutical and Bioactive Natural Products (Attaway, D.H., and Zaborsky, O.R., eds.) Vol. 1, pp. 101–152, Plenum Press, New York.
5. Gerwick, W.H., Nagle, D.G., and Proteau, P.J. (1993) Oxylipins from Marine Invertebrates, in *Topics in Current Chemistry* (Scheuer, P.J., ed.) Vol. 167, pp. 117–180, Springer-Verlag, Berlin.
6. Gerwick, W.H. (1994) Structure and Biosynthesis of Marine Algal Oxylipins, *Biochim. Biophys. Acta 1211*, 243–255.
7. Jacobson, P.B., and Jacobs, R.S. (1992) Fuscoside: An Anti-inflammatory Marine Natural Product Which Selectively Inhibits 5-Lipoxygenase. Part I: Physiological and Biochemical Studies in Murine Inflammatory Models, *J. Pharmacol. Exp. Therapeutics 262*, 866–873.
8. Jacobson, P.B., and Jacobs, R.S. (1992) Fuscoside: An Anti-inflammatory Marine Natural Product Which Selectively Inhibits 5-Lipoxygenase. Part II: Biochemical Studies in the Human Neutrophil, *J. Pharmacol. Exp. Therapeutics 262*, 874–882.
9. de Carvahlo, M.S., and Jacobs, R.S. (1991) Two-Step Inactivation of Bee Venom Phospholipase A_2 by Scalaradial, *Biochem. Pharmacol. 42*, 1621–1626.
10. de Freitas, J.C., Blankemeier, L.A., and Jacobs, R.S. (1984) *In vitro* Inactivation of the Neurotoxic Action of *B*-Bungarotoxin by the Marine Natural Product, Manoalide, *Experimentia 40*, 864–865.
11. Glaser, K.B., and Jacobs, R.S. (1986) Molecular Pharmacology of Manoalide Inactivation of Bee Venom Phospholipase A_2, *Biochem. Pharmacol. 35*, 449–453.
12. Lombardo, D., and Dennis, E.A. (1985) Cobra Venom Phospholipase A_2 Inhibition by Manoalide. A Novel Type of Phospholipase Inhibitor, *J. Biol. Chem. 260*, 7234–7240.
13. Gerwick, W.H., Moghaddam, M.F., and Hamberg, M. (1991) Oxylipin Metabolism in the Red Alga *Gracilariopsis lemaneiformis*: Mechanism of Formation of Vicinal Dihydroxy Fatty Acids, *Arch. Biochem. Biophys. 290*, 436–444.
14. Moore, R.E. (1981) Constituents of Blue-Green Algae, in *Marine Natural Products* (Scheuer, P.J., ed.) Vol. 4, pp. 1–52, Academic Press, New York.
15. Beebe, J.M., Brown, P.R., and Turcotte, J.G. (1989) Analytical-Scale High-Performance Liquid Chromatography of Omega-3 Fatty Acid Esters Derived from Fish Oils, *J. Chromat. 468*, 225–233.
16. Targett, N.M., and Mitsui, A. (1979) Toxicity of Subtropical Marine Algae Using Fish Mortality and Red Blood Cell Hemolysis for Bioassays, *J. Phycol. 15*, 181–185.
17. Stefanov, K., Konkalieva, M., Brechany, E.Y., and Christie, W.W. (1988) Fatty Acid Composition of Some Algae from the Black Sea, *Phytochemistry 27*, 3495–3497.
18. Littler, D.S., and Littler, M.M. (1991) Systematics of *Anadyomene* species (Anadyomenaceae, Chlorophyta) in the Tropical Western Atlantic, *J. Phycol. 27*, 101–118.
19. Lombardi, P. (1990) A Rapid, Safe, and Convenient Procedure for the Preparation and Use of Diazomethane, *Chemistry & Industry 21*, 708.
20. Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*, 248–254.
21. Borgeat, P., Picard, S., Vallerand, P., Bourgoin, S., Odeimat, A., Siros, P., and Poubelle, P.E. (1990) Automated On-Line Extraction and Profiling of Lipoxygenase Products of Arachidonic Acid by High-Performance Liquid Chromatography, in *Methods in Enzymology* (Murphy, R.C., and Fitzpatrick, F.A., eds.) Vol. 187, pp. 98–116, Academic Press, New York.
22. Burgess, J.R., de la Rosa, R.I., Jacobs, R.S., and Butler, A. (1991) A New Eicosapentaenoic Acid Formed from Arachidonic Acid in the Coralline Red Algae *Bossiella orbigniana*, *Lipids 26*, 162–165.
23. Lopez, A., and Gerwick, W.H. (1987) Two New Icosapentaenoic [sic] Acids from the Temperate Red Seaweed *Ptilota filicina* J. Agardh, *Lipids 22*, 190–194.
24. Tulloch, A.P. (1982) ^{13}C Nuclear Magnetic Resonance Spectroscopic Analysis of Seed Oils Containing Conjugated Unsaturated Acids, *Lipids 17*, 544–550.
25. Husain, S., and Devi, K.S. (1993) Separation and Identification of Isomeric Conjugated Fatty Acids by High-Performance Liquid Chromatography with Photodiode Array Detection, *Lipids 28*, 1037–1040.
26. Hamberg, M. (1992) Metabolism of 6,9,12-Octadecatrienoic Acid in the Red Alga *Lithothamnion corallioides:* Mechanism of Formation of a Conjugated Tetraene Fatty Acid, *Biochem. Biophys. Res. Comm. 188*, 1220–1227.

[Received February 7, 1995, and in revised form May 22, 1995; Revision accepted May 22, 1995]

Synthesis of *E,E*-Diene Hydroperoxides *via* Photoisomerization of Protected Hydroperoxides

P.H. Dussault*, I.Q. Lee and C.T. Eary

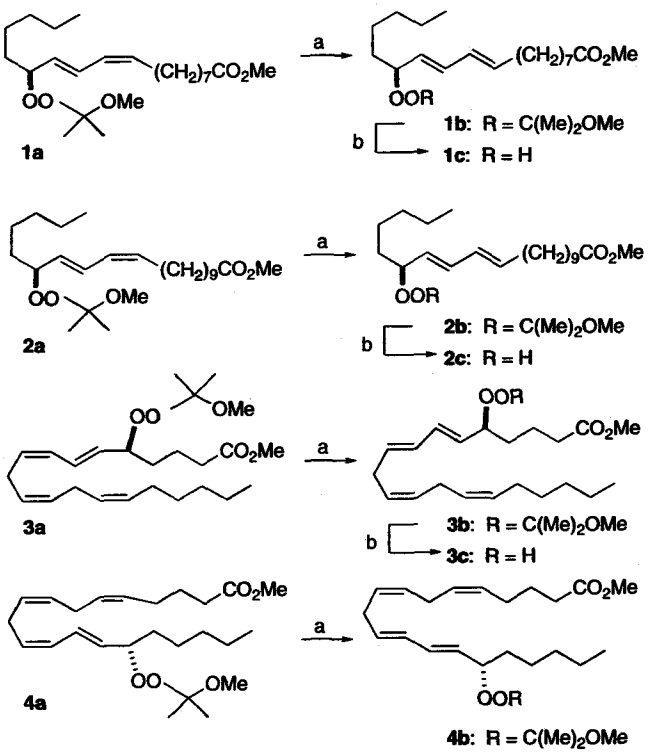
Department of Chemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68588-0304

ABSTRACT: A general approach to *E,E*-diene hydroperoxides is described based upon photoisomerization of readily available *Z,E*-diene monoperoxyketals. Protection of a *Z,E*-diene hydroperoxide as the 2-methoxypropyl peroxyketal is followed by iodine-mediated photoisomerization to produce a mixture enriched in the *E,E* isomer. After chromatographic purification, deprotection of the peroxyketal with mild acid furnishes the *E,E*-diene hydroperoxide.

Lipids 30, 591–594 (1995).

As part of a research program concerned with peroxide synthesis, we became aware of the challenges associated with the synthesis of *E,E*-diene hydroperoxides. Most methods for the construction of diene hydroperoxides, including enzymatic dioxygenation of 1,4-dienes, olefination of γ-peroxy-α,ß-unsaturated aldehydes, or reaction of 1,4-dienes with singlet oxygen, favor production of the *Z,E*-isomers (1–3). Radical autoxidation of 1,4-dienes does produce *E,E*-diene hydroperoxides, but in poor yield and as a mixture of regioisomers (4). The only general method for synthesis of *E,E*-diene hydroperoxides involves silver-mediated addition of hydrogen peroxide to vinylcyclopropanes (5). Given the ready availability of both natural and unnatural *E,Z*-diene hydroperoxides, a method for their conversion to the *E,E*-isomers would represent a useful addition to the existing methodology. We report herein the iodine-catalyzed isomerization of protected *Z,E*-diene hydroperoxides to the *E,E*-isomers [Scheme 1; where a, I_2, CH_2Cl_2, hv; b, $HOAC/H_2O$ (90:10)].

Although radical isomerization has been applied to unsaturated fats and derived alcohols (6,7), extension of this methodology would seem to be ruled out by the reactivity of the hydroperoxide moiety. However, recent reports have demonstrated that conversion of hydroperoxides into monoperoxyketals, a transformation first reported more than forty years ago, imparts greatly increased stability toward a variety of common synthetic protocols (3,8–11). As shown in Scheme 1, we have found that temporary protection of readily available *E,Z*-diene hydroperoxides as their monoperoxyketals allows for chemoselective isomerization of the diene in the presence of the peroxide group.



SCHEME 1

MATERIALS AND METHODS

Reagents. The methyl esters of 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid [13(*S*)-HPOD], 15(*S*)-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid [15(*S*)-HPETE], and 15-hydroperoxy-11*Z*,13*E*-eicosadienoate were prepared according to published procedures (12–14). The monoperoxyketal of 5(*S*)-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid [5(*S*)-HPETE] methyl ester was prepared through total synthesis (3). Halogenated solvents (CH_2Cl_2, $CHCl_3$, and CCl_4) were filtered through a short column of neutral alumina before use. A description of standard protocols for spectral characterization has been reported (3,8).

*To whom correspondence should be addressed.

Abbreviations: EA, ethyl acetate; 13-HPOD, 13(*S*)-hydroperoxy-9*Z*-11*E*-octadecadienoic acid; 15(*S*)-HPETE, 15(*S*)-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; 5(*S*)-HPETE, 5(*S*)-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; NP-HPLC, normal-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance.

Copyright © 1995 by AOCS Press

Ketalization. Peroxyketals were prepared in >90% yield from the reaction of hydroperoxides and 2-methoxypropene in CH_2Cl_2 in the presence of catalytic pyridinium *p*-toluenesulfonate according to a published procedure (8).

Isomerization. I_2 (0.01 equivalent from a 0.1 M stock solution in CCl_4) was added to a stirred 0.3 M solution of the perketal in CH_2Cl_2 or $CHCl_3$. The reaction was stirred for 4–8 h on a bench top under ambient fluorescent lighting (160 W fixture) from a distance of approximately six feet. The concentrated reaction was directly subjected to flash chromatography on silica gel; elution with ethyl acetate (EA)/hexane afforded 70–80% of the diene peroxyketals as an approximately 80:20 *EE/EZ* mixture.

Deprotection. A 0.1 M solution of the diene peroxyketal in HOAc/H_2O (90:10) was stirred for 2 h and then extracted with hexane containing a trace of butylated hydroxytoluene. The hexane layer was washed with water and dried over sodium sulfate. The crude hydroperoxide obtained after concentration was directly subjected to flash chromatography on silica gel; elution with EA/hexane afforded >80% yields of the diene hydroperoxide as a colorless oil. Residual *E,Z*-diene was typically removed at this stage by semi-preparative (25 × 2 cm column) normal-phase high-performance liquid chromatography (NP–HPLC).

Spectral data. Methyl 13(*S*)-[(1-methoxy-1-methylethyl)-dioxy]-9*Z*,11*E*-octadecadienoate (**1a**): R_f = 0.4 in 10% EA/hexane; ^{1}H nuclear magnetic resonance (NMR) (300 MHz) δ 6.45 (*dd*, 1H, *J* = 15.3, 11.3), 5.97 (*t*, 1H, *J* = 10.9), 5.59 (*dd*, 1H, *J* = 15.3, 7.6), 5.42 (*dt*, 1H, *J* = 10.8, 7.7), 4.38 (*q*, 1H, *J* = 7.3), 3.64 (*s*, 3H), 3.27 (*s*, 3H), 2.28 (*t*, 2H, *J* = 7.7), 2.15 (*q*, 2H, *J* = 8.1), 1.6–1.2 (24H), 0.86 (*t*, 3H, *J* = 6.85).

Methyl 13(*S*)-[(1-methoxy-1-methylethyl)dioxy]-9*E*,11*E*-octadecadienoate (**1b**): R_f = 0.4 in 10% EA/hex; ^{1}H NMR (300 MHz) δ 6.16 (*dd*, 1H, *J* = 15.3, 10.5), 6.02 (*dd*, 1H, *J* = 15.0, 10.5), 5.69 (*dt*, 1H, *J* = 14.8, 7.4), 5.50 (*dd*, 1H, *J* = 15.0, 8.1), 4.34 (*q*, 1H, *J* = 7.6), 3.65 (*s*, 3H), 3.28 (*s*, 3H), 2.29 (*t*, 2H, *J* = 7.6), 2.05 (*q*, 2H, *J* = 6.9) 1.62–1.28 (24H), 0.86 (*t*, 3H, *J* = 6.7)

Methyl 13(*S*)-hydroperoxy-9*E*,11*E*-octadecadienoate (**1c**): Following deprotection of **1b**, the mixture of hydroperoxides was purified by NP–HPLC (0.25% ethanol/hexane) to afford spectroscopically pure *E,E*-diene as a colorless oil: R_f = 0.3 in 10% EA/hexane; ^{1}H NMR (300 MHz) δ 7.72 (*s*, 1H) 6.26 (*dd*, 1H, *J* = 15.3, 10.5), 6.04 (*dd*, 1H, *J* = 15.0, 10.5), 5.75 (*dt*, 1H, *J* = 14.5, 7.5), 5.36 (*dd*, 1H, *J* = 15.1, 8.5), 4.38 (*q*, 1H, *J* = 8.1), 3.65 (*s*, 3H), 2.29 (*t*, 2H, *J* = 7.6), 2.07 (*q*, 2H, *J* = 6.9) 1.63–1.25 (18H), 0.86 (*t*, 3H, *J* = 6.7); ^{13}C NMR (125 MHz) δ 137.1, 135.8, 130.0, 129.7, 87.4, 52.1, 34.7, 33.2, 32.4, 29.7, 29.5, 25.6, 25.5, 23.1, 14.6.

Methyl 13(*S*)-hydroxy-9*E*,11*E*-octadecadienoate (**1d**): Methyl ester **1c** (38.7 mg, 0.12 mmol) was dissolved in 3 mL of EA with triphenylphosphine (32.5 mg, 0.12 mmol) and stirred at 25°C for 40 min. The solution was concentrated to dryness and directly subjected to flash chromatography (10% EA/hexane) to afford 31.2 mg (85%) of the hydroxy methyl ester: R_f = 0.1 in 10% EA/hexane; [α]D = +7.23 (c = 0.16, CH_2Cl_2); ^{1}H NMR (500 MHz) δ 6.16 (*dd*, 1H, *J* = 14.9, 10.1), 6.0 (*dd* 1H, *J* = 15.3, 10.5), 5.67 (*dt*, 1H, *J* = 14.5, 6.9), 5.56 (*dd*, 1H, *J* = 15.3, 7.3), 4.1 (*m*, 1H, *J* = 7.3), 3.65 (*s*, 3H), 2.29 (*t*, 2H, *J* = 7.7), 2.04 (*q*, 2H, *J* = 6.9), 1.6–1.25 (18H), 0.87 (*t*, 3H, *J* = 6.9); ^{13}C NMR (125 MHz) δ 135.9, 134.4, 131.5, 130.2, 73.5, 52.0, 37.9, 34.7, 33.2, 32.4, 29.8, 29.7, 29.6, 25.7, 25.5, 23.2, 14.6.

13-(*S*)-Hydroxy-9*E*,11*E*-octadecadienoic acid (**1e**): Methyl ester **1d** (29.7 mg, 0.096 mmol) and K_2CO_3 (132 mg, 0.96 mmol) were dissolved in MeOH/H_2O (4:1) (1.42 mL) and stirred at room temperature for 72 h. The mixture was diluted with 2 mL of H_2O, acidified with 5% HCl, and extracted with 20% EA/hexane. The organic extracts were combined, washed with water, and dried/filtered over $MgSO_4$/Celite. The organic layer was concentrated and directly subjugated to flash chromatography in 225:15:1 hexane/EtOH/HOAc to afford 24.8 mg (85%) of the hydroxy acid: R_f = 0.4 in 225:25:1 hexane/EtOH/HOAc; [α]D = +5.4 (c = 0.8, $CHCl_3$); ^{1}H NMR (500 MHz) δ 6.17 (*dd*, 1H, *J* = 15.0, 10.3), 6.02 (*dd*, 1H, *J* = 15.0, 10.5), 5.70 (*dt*, 1H, *J* = 14.6, 7.1), 5.57 (*dd*, 1H, *J* = 15.0, 7.2), 4.11 (*q*, 1H, *J* = 6.5), 2.35 (*t*, 2H, *J* = 7.3), 2.07 (*q*, 2H, *J* = 7.3, 6.9), 1.64–1.25 (18H), 0.89 (*t*, 3H, *J* = 6.9); ^{13}C NMR (125 MHz) δ 136.0, 134.3, 131.6, 130.2, 73.6, 37.9, 33.2, 32.4, 30.3, 29.7, 29.6, 29.5, 25.8, 23.2, 14.7.

Methyl 15(*S*)-[(1-methoxy-1-methylethyl)dioxy]-11*Z*,13*E*-eicosadienoate (**2a**): R_f = 0.6 in 10% EA/hexane; ^{1}H NMR (300 MHz) δ 6.46 (*dd*, 1H, *J* = 15.3, 11.3), 5.97 (*t*, 1H, *J* = 11.3, 10.9), 5.59 (*dd*, 1H, *J* = 15.3, 8.1), 5.4 (*dt*, 1H, *J* = 10.9, 7.7), 4.4 (*dd*, 1H, *J* = 6.9, 7.3), 3.64 (*s*, 3H), 3.27 (*s*, 3H), 2.28 (*t*, 2H, *J* = 7.7), 2.16 (*dd*, 2H, *J* = 7.3, 6.1), 1.62–1.28 (28H), 0.86 (*t*, 3H, *J* = 7.1).

Methyl 15(*S*)-[(1-methoxy-1-methylethyl)dioxy]-11*E*,13*E*-eicosadienoate (**2b**): R_f = 0.6 in 10% EA/hexane; ^{1}H NMR (300 MHz) δ 6.16 (*dd*, 1H, *J* = 15.3, 10.5), 6.02 (*dd*, 1H, *J* = 15.0, 10.5), 5.69 (*dt*, 1H, *J* = 14.8, 7.4), 5.5 (*dd*, 1H, *J* = 15.1, 8.1), 4.38 (*dd*, 1H), 3.65 (*s*, 3H), 3.28 (*s*, 3H), 2.29 (*t*, 2H, *J* = 7.6), 2.05 (*q*, 2H *J* = 6.9), 1.62–1.28 (28H), 0.86 (*t*, 3H, *J* = 6.7).

Methyl 15(*S*)-hydroperoxy-11*E*,13*E*-eicosadienoate(**2c**): The diene was purified by NP–HPLC (7% EA/hexane). R_f = 0.4 in 10% EA/hexane; ^{1}H NMR (300 MHz) δ 7.72 (*s*, 1H), 6.26 (*dd*, 1H, *J* = 15.3, 10.5), 6.04 (*dd*, 1H, *J* = 15.0, 10.5), 5.75 (*dt*, 1H, *J* = 14.5, 7.5), 5.36 (*dd*, 1H, *J* = 15.1, 8.5), 4.38 (*q*, 1H, *J* = 8.1), 3.65 (*s*, 3H), 2.29 (*t*, 2H, *J* = 7.6), 2.07 (*q*, 2H *J* = 6.9), 1.63–1.25 (22H), 0.86 (*t*, 3H, *J* = 6.7).

Methyl 5(*S*)-[(1-methoxy-1-methylethyl)dioxy]-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoate (**3a**): R_f = 0.6 in 20% EA/hexane. ^{1}H NMR (300 MHz) δ 6.51 (*dd*, 1H, *J* = 15.3, 11.2), 5.98 (*t*, 1H, *J* = 10.9), 5.61 (*dd*, 1H, *J* = 15.3, 8.0), 5.36 (5H), 4.36 (*q*, 1H, *J* = 7.6), 3.64 (*s*, 3H), 3.26 (*s*, 3H), 2.94 (*t*, 2H, *J* = 6.2), 2.79 (*t*, 2H, *J* = 6.0), 2.32 (*t*, 2H, *J* = 7.2), 2.03 (*q*, 2H, *J* = 6.9), 1.69 (2H), 1.55–1.25 (14H), 0.86 (*t*, 3H *J* = 6.8); ^{13}C NMR (75 MHz) δ 173.7, 132.7, 130.6, 130.5, 128.9, 128.0, 127.9, 127.4, 127.3, 104.6, 84.0, 51.4, 49.2, 33.8, 32.5, 31.5, 29.3, 27.2, 26.1, 25.6, 23.0, 22.7, 22.5, 20.9, 14.0; high-resolution

mass spectrometry (HRMS) calculated for $C_{25}H_{42}O_5Li$ (M + Li) 429.3192, found: 429.3210.

Methyl 5(*S*)-[(1-methoxy-1-methylethyl)dioxy]6*E*,8*E*,11*Z*, 14*Z*-eicosatetraenoate (**3b**): The isomerized 5(*S*)-HPETE peroxyketal was purified by NP–HPLC (7% EA/hexane) to afford the spectroscopically pure 6*E*,8*E*,11*Z*,14*Z*-isomer (30 mg, 72%) as a colorless oil: R_f = 0.6 in 20% EA/hexane; $[\alpha]_D$ = -22.2 (c = 0.36); ^{1}H NMR (300 MHz) δ 6.19 (*dd*, 1H, *J* = 15.3, 10.4), 6.07 (*dd*, 1H, *J* = 15.0), 5.68 (*dt*, 1H, *J* = 15.0, 7.6), 5.52 (*dd*, 1H, *J* = 15.0, 8.1), 5.40 (4H), 4.36 (*q*, 1H, *J* = 7.6), 3.65 (*s*, 3H), 3.27 (*s*, 3H), 2.85 (*t*, 2H, *J* = 6.2), 2.78 (*t*, 2H, *J* = 6), 2.32 (*t*, 2H, *J* = 7.2), 2.03 (*q*, 2H, *J* = 6.9), 1.69 (2H), 1.58 (*s*, 6H); 1.35–1.25 (8H), 0.86 (*t*, 3H, *J* = 6.8); ^{13}C NMR (75 MHz) δ 173.8, 133.3, 133.2, 130.5, 130.4, 129.8, 129.4, 127.4, 126.6, 104.6, 84.1, 51.5, 49.3, 33.9, 32.5, 31.5, 30.4, 27.3, 27.2, 25.6, 23.0, 22.8, 22.5, 20.9, 14.0.

Methyl 5(*S*)-hydroperoxy-6*E*,8*E*,11*Z*,14*Z*-eicosatetraenoate (**3c**): R_f = 0.4 in 20% EA/hexane; ^{1}H NMR (300 MHz) δ 7.94 (*s*, 1H), 6.28 (*dd*, 1H, *J* = 15.3, 10.5), 6.04 (*t*, 1H, *J* = 15.0), 5.74 (*dt*, 1H, *J* = 15.0, 7.6), 5.52 (*dd*, 1H, *J* = 15.0, 8.1), 5.40 (4H), 4.42 (*q*, 1H, *J* = 7.6), 3.66 (*s*, 3H), 2.85 (*t*, 2H, *J* = 6.2), 2.78 (*t*, 2H, *J* = 6.2), 2.32 (*t*, 2H, *J* = 6.8), 2.04 (*q*, 2H, *J* = 6.8), 1.76–1.28 (10H), 0.88 (*t*, 3H, *J* = 6.8, Me).

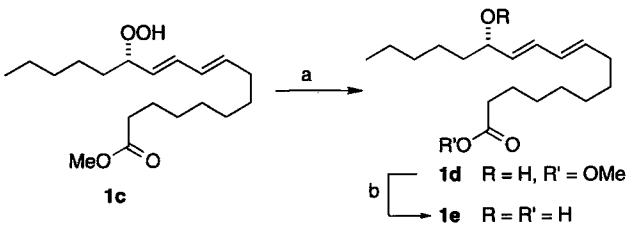
Methyl 15(*S*)-[(1-methoxy-1-methylethyl)dioxy]-5*Z*,8*Z*,11*Z*, 13*E*-eicosatetraenoate (**4a**): R_f = 0.5 in 10% EA/hexane; ^{1}H NMR (300 MHz) δ 6.50 (*dd*, 1H, *J* = 15.3, 10.9), 6.00 (apparent triplet, 1H, *J* = 10.7), 5.64 (*dd*, 1H, *J* = 15.1, 8.0), 5.37 (*m*, 5H), 4.40 (*q*, 1H, *J* = 7.6), 3.66 (*s*, 3H), 3.28 (*s*, 3H), 2.95 (*t*, 2H, *J* = 6), 2.79 (*t*, 2H, *J* = 5.5), 2.30 (*t*, 2H, *J* = 7.6), 2.10 (*qt*, 2H, *J* = 7), 1.69 (*quintet*, 2H, *J* = 7.4), 1.46–1.25 (14H), 0.87 (*t*, 3H, *J* = 6.5); ^{13}C NMR (75 MHz) δ 174.1, 133.6, 130.1, 129.0, 128.7, 128.6, 128.2, 127.6, 127.5, 104.6, 84.7, 51.5, 49.2, 33.4, 33.0, 31.7, 26.5, 26.1, 25.6, 25.1, 24.7, 23.0, 22.8, 22.5, 14.1; high-resolution mass spectrometry calcd. for $C_{25}H_{42}O_5Li$ (M + Li) 429.3193, found 429.3207.

Methyl 15(*S*)-[(1-methoxy-1-methylethyl)dioxy]-5*Z*,8*Z*,11*E*, 13*E*-eicosatetraenoate (**4b**) was purified by NP–HPLC (7% EA/hexane): R_f = 0.6 in 20% EA/hexane; ^{1}H NMR (300 MHz) δ 6.19 (*dd*, 1H, *J* = 15.0, 10.4), 6.07 (*dd*, 1H, *J* = 14.8, 10.5), 5.68 (*dt*, 1H, *J* = 14.6, 6.4), 5.56 (*dd*, 1H, *J* = 15.0, 8.1), 5.40 (4H, olefinic), 4.34 (*q*, 1H, *J* = 7.6), 3.65 (*s*, 3H), 3.27 (*s*, 3H), 2.85 (*t*, 2H, *J* = 5.6), 2.78 (*t*, 2H, *J* = 6.2), 2.31 (*t*, 2H), 2.08 (*q*, 2H, *J* = 6.7), 1.69 (*quintet*, 2H, *J* = 7.4), 1.57–1.21 (14H), 0.86 (*t*, 3H, *J* = 6.8).

RESULTS AND DISCUSSION

This strategy was initially investigated with a monoperoxyketal (**1a**) ultimately derived from lipoxygenase-mediated dioxygenation of linoleic acid. No isomerization occurred upon either addition of 0.5 equivalent of diphenyldisulfide or brief photolysis with a Pyrex-filtered (λ ≥ 320 nm), medium-pressure Hg lamp, and photolysis of a deoxygenated diene/disulfide mixture led to simultaneous isomerization and decomposition. The apparent incompatibility between the monoperoxyketal and the intermediate thiyl radical led us to investigate the use of a milder catalyst. Addition of 1 mol% of I_2 to a $CHCl_3$ or CH_2Cl_2 solution of the perketal under fluorescent room lights led to diene equilibration. Prolonged reaction times or use of a more powerful light source resulted in elimination to a dienone. No reaction was observed in the dark. NMR analysis revealed an equilibrium mixture comprising approximately 80:20 mixture of the 9*E*,11*E*/9*E*,11*Z* isomers of monoperoxyketal **1b**. Acidic deprotection afforded a mixture of 9*E*,11*E*- and 9*Z*,11*E*-diene hydroperoxides. Similar results were observed for the isomerization of an eicosadienoate monoperoxyketal (**2a**).

HPLC purification of the mixture of hydroperoxides derived from **1b** afforded optically active 9*E*,11*E*-13-HPOD methyl ester (**1c**), which was converted to the enantiomer of coriolic acid as shown in Scheme 2 (15) [where a, PPh_3, EtOAc (85%); b, K_2CO_3, $MeOH/H_2O$ (85%)]. Comparison of the optical rotations indicate the enantiomeric excess of **1e**, and therefore **1c**, to be >90%; clearly, the isomerization does not affect the hydroperoxide-bearing carbon.



SCHEME 2

We next attempted application of this methodology to polyene hydroperoxides, substrates containing both conjugated and nonconjugated diene units. The monoperoxyketal of 5(*S*)-HPETE methyl ester (**3a**) underwent selective isomerization of the 6,8-diene to afford *E*,*E*-monoperoxyketal **3b**, which was deprotected to furnish the 8*E*-isomer of 5(*S*)-HPETE methyl ester (**3c**). Similarly, the monoperoxyketal of 15(*S*)-HPETE methyl ester (**4a**) underwent selective isomerization of the 11,13-diene to produce the 11*E* isomer **4b**. The lack of change in the ^{1}H NMR signals for the isolated *bis*allylic methylene groups (C_{13} in **3b** or C_7 in **4b**) verified the lack of isomerization at the nonconjugated diene subunits.

In summary, the reversible masking of diene hydroperoxides as their methoxypropyl peroxyketals is found to allow for chemoselective isomerization of the conjugated diene to produce *E*,*E*-diene hydroperoxides.

REFERENCES

1. Veldink, G.A., and Vliegenthart, J.F.G. (1984) in *Advances in Inorganic Biochemistry*, (Eichorn, G.L., and Mazilli, L.G., eds.) Vol. 6, pp. 139–161, Elsevier, New York.
2. Porter, N.A., Logan, J., and Kontoyiannidou, V. (1979) Preparation and Purification of Arachidonic Acid Hydroperoxides of Biological Importance, *J. Org. Chem. 44*, 3177–3181.

3. Dussault, P., and Lee, I.Q. (1995) A Chemoenzymatic Approach to Hydroperoxyeicosatetaenoic Acids (HPETEs): Total Synthesis of 5(*S*)–HPETE, *J. Org. Chem. 60*, 218–226.
4. Porter, N.A. (1986) Mechanisms for the Autoxidation of Polyunsaturated Lipids, *Acc. Chem. Res. 19*, 262–268.
5. Porter, N.A., Roberts, D.H., and Ziegler, C.B.J. (1980) A New Route to Lipid Hydroperoxides: Orbital Symmetry controlled Ring Opening of Vinylcyclopropyl Bromides, *J. Am. Chem. Soc. 102*, 5913–5915.
6. Gunstone, F.D., and Ismail, I.A. (1967) Fatty Acids. Part 14. The Conversion of the *cis*–Octadecadienoic Acids to Their *Trans*–Isomers, *Chem. Phys. Lipids 1*, 264–269.
7. Canaguier, R., Cecchi, G., Ucciani, E., Canaguier, R., and Chevalier, J.L. (1984) Photochemical Isomerization of Polyunsaturated Fatty Acids Catalyzed by Iodine, *Rev. Fr. Corps Gras 31*, 401–409.
8. Dussault, P., Sahli, A., and Westermeyer, T. (1993) An Organometallic Approach to Peroxyketals, *J. Org. Chem. 58*, 5469–5474.
9. Porter, N.A., Dussault, P., Breyer, R.A., Kaplan, J., and Morelli, J. (1990) The Resolution of Racemic Hydroperoxides: A Chromatography-Based Separation of Perketals Derived from Arachidonic, Linoleic and Oleic Acid Hydroperoxides, *Chem. Res. Toxicol. 3*, 236–242.
10. Rigaudy, J., and Izoret, G. (1953) Addition of Hydroperoxides to Activated Double Bonds of Vinyl Ethers, *C. R. Hebd. Seances Acad. Sci. 236*, 2086–2088.
11. Milas, N.A., Peeler, R.L., and Mageli, O.L. (1954) Organic Peroxides. XIX. α-Hydroperoxyethers and Related Peroxides, *J. Am. Chem. Soc. 76*, 2322–2325.
12. Iacazio, G., Langr, G., Baratti, J., Buono, G., and Triantaphylides, C. (1990) Preparative, Enzymatic Synthesis of Linoleic Acid (13*S*)–Hydroperoxide Using Soybean Lipoxygenase–1, *J. Org. Chem. 55*, 1690–1691.
13. Baldwin, J.E., Davies, D.I., Hughes, L., and Gutteridge, N.J.A. (1979) Synthesis from Arachidonic Acid of Potential Prostaglandin Precursors, *J. Chem. Soc., Perkin Trans. I*, 115–121.
14. Dussault, P., and Lee, I.Q. (1992) The Total Synthesis of 15(*S*)–HPETE, *J. Org. Chem. 57*, 1952–1954.
15. Kobayashi, Y., Okamoto, S., Shimazaki, T., Ochiai, Y., and Sato, F. (1987) Synthesis and Physiological Activities of Both Enantiomers of Coriolic Acid and Their Geometric Isomers, *Tetrahedron Lett. 28*, 3959–3962.

[Received March 6, 1995, and in revised form April 14, 1995; Revision accepted April 20, 1995]

Furan Fatty Acids Determined as Oxidation Products of Conjugated Octadecadienoic Acid

Martin P. Yurawecz*, Jennifer K. Hood, Magdi M. Mossoba, John A.G. Roach and Yuoh Ku

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC 20204

ABSTRACT: The objective of this study was to identify oxidation products of conjugated linoleic acid (CLA), a series of octadecadienoic acids with conjugated double bonds, which have been reported to have antioxidant and anticarcinogenic properties. Reference materials of CLA were oxidized in different concentrations of water/methanol; for example, 0.5 g octadecadienoic acid was dissolved in 50 mL methanol, and 100 mL water was added; this suspension was heated at 50°C and continuously aerated. Aliquots of 5 mL were taken over time, extracted with ether, treated with diazomethane and examined by gas chromatography/mass spectrometry and/or gas chromatography with flame-ionization detection. Products identified included the following furan fatty acids (FFAs): 8,11-epoxy-8,10-octadecadienoic; 9,12-epoxy-9,11-octadecadienoic; 10,13-epoxy-10,12-octadecadienoic; and 11,14-epoxy-11,13-octadecadienoic. Conjugated dienes should be considered as a possible source of FFAs, and CLA may have products common to furans in their overall oxidative scheme.

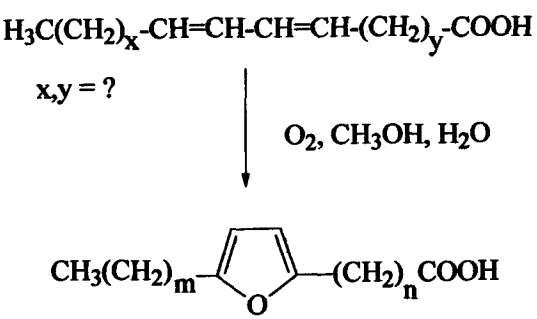
Lipids 30, 595–598 (1995).

"Conjugated linoleic acid" (CLA) is a term used to describe a group of octadecadienoic acids which contain two conjugated double bonds. Interest in CLA has been growing as a result of recently reported antioxidant (1) and anticarcinogenic (1–3) properties of these compounds.

It is not apparent from the structure of CLA isomers how these compounds could be effective antioxidants. CLA does not have a tautomeric form nor does it seem to have the functional group necessary to chelate metal ions. The oxidation products of conjugated fatty acids have been studied much less than their methylene-interrupted counterparts (4).

Previous oxidation studies of conjugated acids do not indicate that hydroxylation occurs to the extent that significant stable products are formed (4). The work described here attempted to characterize oxidation products of CLA by oxidizing a commercially obtained reference, nominally 9,11-octadecadienoic acid, a mixture of *cis* and *trans* isomers of an octadecadienoic acid.

Initial attempts to oxidize CLA in a methanol solution were unsuccessful; the soluble CLA was remarkably stable, e.g., there were no detectable products after days of attempted oxidation at 45°C. When enough water was added to produce a suspension, oxidation commenced. The oxidation then proceeded at a controlled and reproducible rate. Four furan fatty acids (FFAs), 8,11-epoxy-8,10-octadecadienoic ($F_{8,11}$); 9,12-epoxy-9,11-octadecadienoic ($F_{9,12}$); 10,13-epoxy-10,12-octadecadienoic ($F_{10,13}$); and 11,14-epoxy-11,13-octadecadienoic ($F_{11,14}$), as shown in Scheme 1, were identified by gas chromatography/mass spectrometry (GC/MS) and GC/matrix isolation (MI)/Fourier transform–infrared spectroscopy (FT–IR) when methylated ether extracts of an oxidized, commercially obtained CLA reference were examined by GC.



	m	n
$F_{8,11}$	6	6
$F_{9,12}$	5	7
$F_{10,13}$	4	8
$F_{11,14}$	3	9

SCHEME 1

*To whom correspondence should be addressed at the Division of Science and Applied Technology, HFS-175, Food and Drug Administration, 200 C Street, S.W., Washington, DC 20204.

Abbreviations: CLA, conjugated linoleic acid; EI, electron ionization; $F_{8,11}$, 8,11-epoxy-8,10-octadecadienoic acid; $F_{9,12}$, 9,12-epoxy-9,11-octadecadienoic acid; $F_{10,13}$, 10,13-epoxy-10,12-octadecadienoic acid; $F_{11,14}$, 11,14-epoxy-11,13-octadecadienoic acid; FAME, fatty acid methyl ester; FFA, furan fatty acid; FID, flame-ionization detection; FT–IR, Fourier transform–infrared spectroscopy; GC/MS, gas chromatography/mass spectrometry; MI, matrix isolation; PFK, perfluorokerosene; SFC, supercritical fluid chromatography.

Copyright © 1995 by AOCS Press

The compound $F_{9,12}$ has been found in *Exocarpus cupressiformis* as a plant lipid (5), and its oxidation and that of $F_{10,13}$ have been studied (6,7). FFAs with alkyl ring substitution have been found in latex (8), fish (9,10), plants (11), and bovine liver (12). The origin (13) and biological function (14,15) of their occurrence are active areas of current research. Although many compounds may result from the oxidation of FFAs (6) and undoubtedly other oxidation products of CLA, this work presents analytical data related only to the identification of the FFAs shown in Scheme 1.

MATERIALS AND METHODS

Diazomethane was prepared from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) according to Technical Information Bulletin No. AL-180, Aldrich Chemical Company (Milwaukee, WI). CLA, 9,11-octadecadienoic acid (manufacturer's assay: *c*-9,*t*-11/*t*-9,*c*-11 41%; *t*-10,*c*-12 44%; *c*-10,*c*-12 9.5%; *t*-9,*t*-11/*t*-10,*t*-12 1.3%; *c*-9,*c*-11 1%; *c*-9,*c*-12 0.7%) and other fatty acids and methyl esters were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). CLA produced from alkali isomerization of safflower oil (assay: palmitic/oleic/stearic 30%; linoleic 0.5%; *c*-9,*t*-11-$C_{18:2}$/*t*-9,*c*-11-$C_{18:2}$ 29%; *t*-10,*c*-12-$C_{18:2}$ 32%; *c*-9,*c*-11-$C_{18:2}$ 2.4%; *c*-10,*c*-12-$C_{18:2}$ 2.7%; *t*-9,*t*-11-$C_{18:2}$/*t*-10,*t*-12-$C_{18:2}$ 4.8%) was obtained from Sou F. Chin (Food Research Institute, University of Wisconsin, Madison, WI). Organic solvents were redistilled-from-glass. Water was distilled and deionized. Cylinder air was breathing quality. Methyl $F_{9,12}$ was synthesized by a previously published procedure (16).

CLA oxidation. Stearic acid ($C_{18:0}$) or heptadecanoic acid ($C_{17:0}$) was sometimes used as the internal reference for the oxidations described below. CLA acids were exposed to varying amounts of air, water, and methanol. Several oxidations were performed similarly, with the following minor variations: *Oxidation 1* (OX_1): Fixed quantities of 75 mg CLA and 3.38 mg $C_{17:0}$ were dissolved in 1 mL methanol in test tubes; 0.0, 0.1, 0.2, 0.5, 1.0, or 2 mL water was added and the individual tubes, which contained air, were sealed and immersed in an oil bath and held at 45°C for 46 h. *Oxidation 2* (OX_2): 0.5 g CLA was dissolved in 50 mL methanol in a 250-mL Erlenmeyer flask with a stirring bar and air flowing through a pipette under the liquid surface at 100–200 mL/min. Then 100 mL water was added, and the suspension was heated on a stirrer-hot plate at temperatures varying from 48 to 69°C. The heat was turned off after 7.5 h, but restored 16 h later. Methanol/water (1:2) was added as needed to maintain a volume between 100 and 150 mL. Aliquots (5 mL) were taken hourly and examined after methylation. *Oxidation 3* (OX_3): Fixed quantities (e.g., 44.2 mg CLA and 3.22 mg $C_{18:0}$) were dissolved in 2 mL methanol in test tubes; a micro stirring bar and 4 mL water were added to each tube. The tubes were placed in an oil bath on a stirrer-hot plate and held at 40°C. Tubes were continuously stirred and aerated (2–5 mL/min). Methanol/water (1:2) was added as needed to maintain a volume between 4 and 6 mL. Individual tubes were removed from the oil bath at different times and examined after methylation.

Methylation procedure. To each aliquot in a test tube were added 5 mL saturated NaCl/water and 5 mL ethyl ether. The tube was vortexed for 30–60 s. The ether portion was dried over Na_2SO_4; 1 mL diazomethane in ether solution was carefully and slowly added to the dried ether portion. After 20 min, 1–2 drops of acetic acid were added to react with the remaining diazomethane. The solution was injected into the gas chromatograph without further cleanup.

Instrumentation. For GC, a Hewlett-Packard 5890A instrument was used under the following conditions: column, 50 m × 0.25 mm i.d. CP Sil 88 capillary (Chrompack, Raritan, NJ); helium carrier gas; flame-ionization detector (FID); temperatures, injector 220°C, detector 280°C, column 75°C for 2 min, then raised 20°C/min to 185°C and held for 33 min, then raised at 4°C/min to 225°C. Samples were run in both split and splitless modes.

Low-resolution electron ionization (EI) GC/MS analyses were obtained with a Hewlett-Packard 5890 series II gas chromatograph coupled to a Fissons VG (Wytheshawe, United Kingdom) Autospec Q mass spectrometer and OPUS 2000 data system. The GC/MS system used version 1.6C software.

The same capillary GC column was used to obtain FID data and GC/MS data. Adjusting the capillary GC column head pressure to 10 psi gave chromatography results comparable to the GC/FID data. The GC/MS conditions were as follows: splitless injection with helium sweep restored 1 min after injection; injector and transfer lines 250°C; oven 75°C for 2 min after injection, then 20°C/min to 185°C, hold 185°C 15 min, 4°C/min to 225°C, hold 225°C 5 min.

The mass spectrometer was tuned to a resolution of 1000 (5% valley) by observing *m/z* 305 in the EI mass spectrum of perfluorokerosene (PFK). The mass scale was calibrated with PFK for magnet scans from 440 to 44 daltons at 1 s per decade. Filament emission was 200 μA at 70 eV. Ion source temperature was 250°C. A TSQ-46 instrument was used for direct probe MS/MS by a procedure that has been described (17).

A Mattson Instruments Model Sirius 100 FT-IR spectrometer equipped with an MI Cryolect interface operated at 10 K under vacuum was used with GC to obtain IR spectra. This system, which was used with a CP-Sil-88 capillary column, has been described in detail (18).

The supercritical fluid chromatography (SFC)/FT–IR system has been previously described in detail (19). Spectra were obtained by using a 25% cyanopropylsiloxane column, 100 μm i.d., with the following density (CO_2) gradient: 0.18 g/mL, hold for 20 min, then from 0.18 to 0.80 g/mL at 0.02 g/mL/min, hold for 5 min. Test solutions were injected from the OX_2 experiment without prior methylation.

RESULTS AND DISCUSSION

The oxidized CLA was treated with diazomethane to obtain methyl esters prior to GC analysis. In initial experiments, it was observed that CLA was very stable in methanol solutions

and the experiment OX_1 was performed to determine if the addition of water would speed up oxidation. No FFAs or other volatile compounds were detected when methanol or 10% water/methanol was used as the solvent. The amounts of FFA produced with increasing percentages of water are as follows: 1.9 mg FFA with 20% water; 2.8 mg FFA with 33% water; 3.0 mg FFA with 50% water; and 2.3 mg FFA with 67% water. It was observed that the better the suspension of CLA acid was, the greater the yield of FFAs.

The chromatogram in Figure 1 shows the FFAs produced after 26.5 h of oxidation by the OX_1 procedure. The major components eluting before CLA include aldehydes, methylated short-chain fatty acids and aldehyde-acids. The FFA esters were identified by mass spectrometry. The general scheme for identifying FFA methyl esters has been reported (20). The molecular ion, *m/z* 308, and the furanyl ring with 0, 1, or 2 methylene groups, *m/z* 67, 81, and 95, respectively, are characteristic of the fatty acid methyl esters (FAMEs) of all the FFAs shown in Scheme 1. The ions characteristic of the specific furan isomers, $[M^+\text{-}(CH_2)_{n-1}CO_2CH_3]$ and $[M^+\text{-}(CH_2)_{m-1}CH_3]$, respectively, and their intensities in parentheses are as follows: $F_{8,11}$, *m/z* 179(100), *m/z* 223(20); $F_{9,12}$, *m/z* 165(100), *m/z* 237(18); $F_{10,13}$, *m/z* 151(100), *m/z* 251(18); and $F_{11,14}$, *m/z* 137(100), *m/z* 265(14). The identity of the FFA methyl ester $F_{9,12}$ was confirmed by comparison of its GC retention time and mass spectrum with those of a synthesized reference material (16).

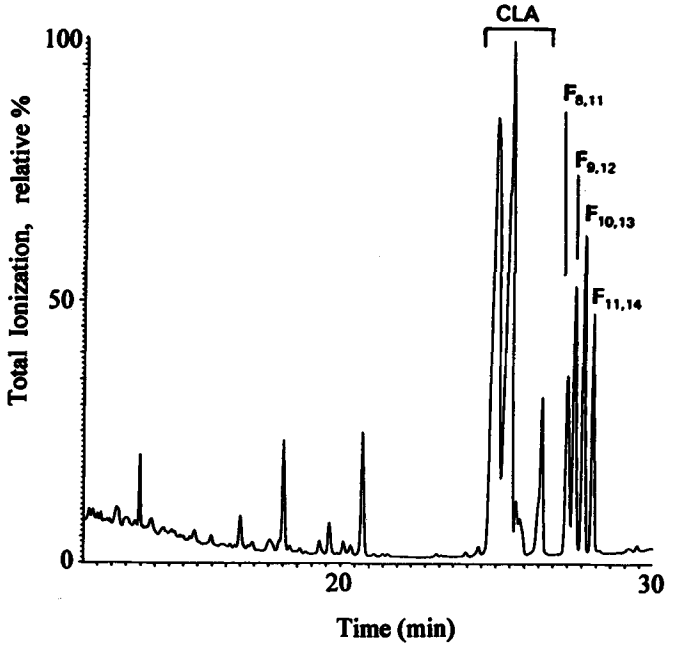


FIG. 1. Chromatogram with total ion mass spectrometry detection after 26.5 h of oxidation of conjugated linoleic acid (CLA) isomers by the Oxidation 2 method (details in Materials and Methods section). Retention regions where methyl esters of CLA and furan fatty acids elute are indicated; earlier responses are due to aldehydes and short-chain fatty acid methyl esters (FAMEs) and aldehyde-acid FAMEs. Methyl esters of $F_{8,11}$, 8,11-epoxy-8,10-octadecadienoic acid; $F_{9,12}$, 9,12-epoxy-9,11-octadecadienoic acid; $F_{10,13}$, 10,13-epoxy-10,12-octadecadienoic acid; $F_{11,14}$, 11,14-epoxy-11,13-octadecadienoic acid.

GC/MI/FT–IR spectra were similar for the CLA oxidation products and the synthesized $F_{9,12}$. The frequencies (4 cm^{-1} resolution) characteristic of the furan that we observed were assigned as follows: 3111, 3031, and 3000 cm^{-1} for =C–H stretch; 1574 cm^{-1} for C=C stretch; 1013 cm^{-1} for C–O stretch; and 780 cm^{-1} for =C–H out-of-plane bend.

The increase in FFA and the decrease in CLA concentration with time (OX_3) are shown in Figure 2. At 46 h, the total of the furan FAMEs amounted to 3.2% of the starting amount of CLA used in this experiment. It is clear from the plot (Fig. 2) that the majority of the mass balance is not accounted for by formation of furans and loss of CLA alone. A plausible explanation, which we intend to investigate, is that under these oxidative conditions the furans are less stable than the CLA isomers from which they may arise. Aldehydes and esters of short- and medium-chain fatty acids and aldehyde-acids may account for a majority of the remaining unaccounted mass, but no effort was made to recover them from the oxidation mixtures.

The CLA produced from alkali isomerization of safflower oil, when oxidized (OX_2), produced only $F_{9,12}$ and $F_{10,13}$. The compositions of the two CLA reference materials that we oxidized differ in the distribution of octadecadienoic acid *cis/trans* isomers. Both reference materials were produced by alkali isomerization of the linoleic fatty acid moiety. The Nu-Chek-Prep assay was performed for only one lot and may not necessarily represent the actual isomeric distribution in the test portion that we examined. It is possible that the FFAs produced may be better indicators of the original CLA composition; hence, in Scheme 1, m = x and n = y. At this time, we do not know how the furans are formed, and the results of these experiments should not be freely extrapolated to other matrices containing CLA.

Researchers have previously shown that FFAs may arise from precursor compounds (e.g., methyl-9,12-dioxo-stearate) when the precursor is injected into a gas chromatograph (10).

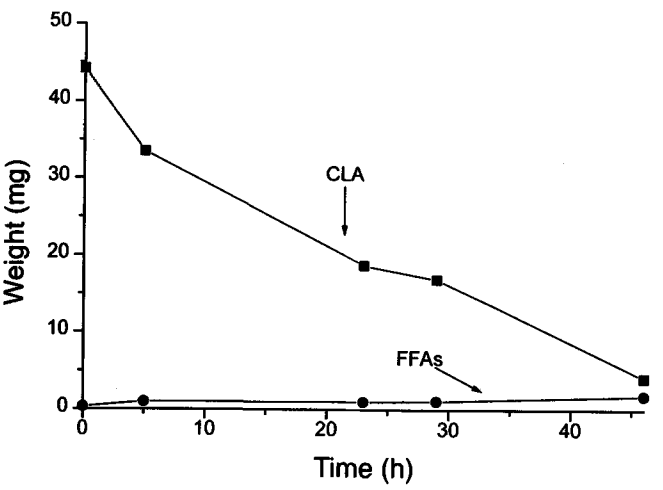


FIG. 2. Abundance of conjugated linoleic acid (CLA) and furan fatty acid (FFA) methyl esters as a function of oxidation time at 40°C, Oxidation 3 method (details in Materials and Methods section); weight is based on stearic acid, which was included in the oxidation.

Furans are also known to arise as a result of dehydration of endoperoxides (21). We do not know the extent to which such precursors may be present in the CLA oxidation mixtures; however, the presence of the furans in methylated and unmethylated CLA oxidation mixtures was confirmed. The presence of furan acids in the unmethylated OX_2 mixture was confirmed by SFC/FT–IR; the major bands expected for the furan acids were observed, and the –C=C– stretch at 1574 cm^{-1} was especially characteristic. The methyl esters of the four FFAs shown in Figure 1 were also confirmed to be present in the methylated, oxidized mixture by direct probe MS/MS. The ions *m/z* 137, 151, 165, and 179 were determined to be daughter ions of the *m/z* 308 methyl FFA parent ion.

It turns out that the same reaction products described in the above experiments are produced by merely exposing neat CLA or CLA methyl esters to ambient air. The greatly improved stability of CLA in solution may be related to its antioxidant properties, i.e., it will readily react, when solvated, only with oxidants that are more reactive than those found in ambient air. The formation of FFAs from conjugated diene systems is known to occur when singlet oxygen is used as a reactant (21). It was surprising that FFAs were produced in the experiments described here, because singlet oxygen was not deliberately introduced into any of the systems described. Oxidation products of conjugated dienes should be considered as a source of FFAs in biological systems.

Experiments are ongoing to establish the mass balance in the described oxidations, and to determine the relationship of the FFAs produced to the specific isomers of octadecadienoic acid used as starting materials.

ACKNOWLEDGMENTS

The authors thank Annette Troy and Elizabeth Calvey of the Food and Drug Administration, Center for Food Safety and Applied Nutrition, for their assistance with the preparation of diazomethane and SFC analysis, respectively.

REFERENCES

1. Ha, Y.L., Storkson, J., and Pariza, M.W. (1990) Inhibition of Benzo(*a*)pyrene-Induced Mouse Forestomach Neoplasia by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 50,* 1097–1101.
2. Ip, C., Chin, S.F., Scimeca, J.A., and Pariza, M.W. (1991) Mammary Cancer Prevention by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 51,* 6118–6124.
3. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1987) Anticarcinogens from Fried Ground Beef: Heat-Altered Derivatives of Linoleic Acid, *Carcinogenesis 8,* 1881–1887.
4. Hopkins, C.Y. (1972) Fatty Acids with Conjugated Unsaturation, in *Topics in Lipid Chemistry* (Gunstone, F.D., ed.) Vol 3., pp. 68–71, John Wiley and Sons Inc., New York.
5. Morris, L.J., Marshall, M.O., and Kelly, V. (1966) A Unique Furanoid Fatty Acid from *Exocarpus* Seed Oil, *Tetrahedron Lett. 36,* 4249–4253.
6. Rosenblat, G., Tabak, M., Lie Ken Jie, M.S.F., and Neeman, I. (1993) Inhibition of Bacterial Urease by Autoxidation of Furan C-18 Fatty Acid Methyl Ester Products, *J. Am. Oil Chem. Soc. 70,* 501–505.
7. Boyer, R.F., Litts, D., Kostishak, J., Wijesundera, R.C., and Gunstone, F.D. (1979) The Action of Lipoxygenase-1 on Furan Derivatives, *Chem. Phys. Lipids 25,* 237–246.
8. Hasma, H., and Subramaniam, A. (1978) The Occurrence of a Furanoid Fatty Acid in *Hevea brasiliensis* Latex, *Lipids 13,* 905–907.
9. Glass, R.L., Krick, T.P., Olson, D.L., and Thorson, R.L. (1977) The Occurrence and Distribution of Furan Fatty Acids in Spawning Male Freshwater Fish, *Lipids 12,* 828–836.
10. Ishil, K., Okajima, H., Koyamatsu, T., Okada, Y., and Watanabe, H. (1988) The Composition of Furan Fatty Acids in the Crayfish, *Lipids 23,* 694–700.
11. Hannemann, K., Puchta, V., Simon, E., Ziegler, H., Ziegler, G., and Spiteller, G. (1989) The Common Occurrence of Furan Fatty Acids in Plants, *Lipids 24,* 296–298.
12. Schödel, R., and Spiteller, G. (1986) Über das Vorkommen von F-Säuren in Rinderleber und deren enzymatischen Abbau bei Gewebeverletzung (The Occurrence of F-Acids in Cattle Liver and Their Enzymatic Degradation During Tissue Damage), *Liebigs Ann. Chem. 5,* 459–462.
13. Gorst-Allman, C.P., Puchta, V., and Spiteller G. (1988) Investigations of the Origin of the Furan Fatty Acids (F-acids), *Lipids 23,* 1032–1036.
14. Sand, D.M., Glass, R.L., Olson, D.L., Pike, H.M., and Schlenk, H. (1984) Metabolism of Furan Fatty Acids in Fish, *Biochim. Biophys. Acta 793,* 429–434.
15. Batna, A., and Spiteller, G. (1994) Effects of Soybean Lipoxygenase-1 on Phosphatidylcholines Containing Furan Fatty Acids, *Lipids 29,* 397–403.
16. Lie Ken Jie, M.S.F., Chan, H.W.M., Wai, J.S.M., and Sinha, S. (1981) Fatty acids: XXIII. A Rapid Method for the Preparation of C_{18} Furanoid Fatty Ester Involving Dry-Column Chromatography, *J. Am. Oil Chem. Soc. 50,* 705–706.
17. Roach, J.A.G., and Carson, L.J. (1987) Collisionally Activated Decomposition Mass Spectrometry/Mass Spectrometry Determination of Organophosphorus Pesticide Residues in Foods, *J. Assoc. Off. Anal. Chem. 70,* 439–442.
18. Mossoba, M.M., Niemann, R.A., and Chen, J.-Y.T. (1989) Picogram Level Quantitation of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Fish Extracts by Capillary Gas Chromatography/Matrix Isolation/Fourier Transform Infrared Spectrometry, *Anal. Chem. 61,* 1678–1685.
19. Calvey, E.M., McDonald, R.E., Page, S.W., Mossoba, M.M., and Taylor, L.T. (1991) Evaluation of SFC/FT-IR for Examination of Hydrogenated Soybean Oil, *J. Agric. Food Chem. 39,* 542–548.
20. McCloskey, J.A. (1969) Mass Spectrometry of Fatty Acid Derivatives, in *Topics in Lipid Chemistry* (Gunstone, F.D., ed.) Vol. 1, pp. 404–405, John Wiley and Sons Inc., New York.
21. Ideses, R., Shani, A., and Klug, J.T. (1982) Cyclic Peroxide: an Isolable Intermediate in Singlet Oxygen Oxidation of Pheromones to the Furan System, *Chem. Ind. 19,* 409–410.

[Received November 9, 1995, and in revised form April 6, 1995; Revision accepted April 10, 1995]

Reinvestigation of the Antioxidant Properties of Conjugated Linoleic Acid

Jeroen J.M. van den Berg[a,*], Nancy E. Cook[a] and Diane L. Tribble[b]

[a]Children's Hospital Oakland Research Institute, Oakland, California 94609 and [b]Department of Molecular and Nuclear Medicine, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

ABSTRACT: Despite repeated suggestions that antioxidant activity of conjugated linoleic acid (CLA), a collective of conjugated dienoic isomers of linoleic acid, underlies its reported anticarcinogenic and antiatherosclerotic effects, the antioxidant properties of CLA remain ill-defined. Therefore, this study was undertaken to gain more insight into the mechanism of potential CLA antioxidant activity. It was tested whether CLA could protect membranes composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) from oxidative modification under conditions of metal ion-dependent or -independent oxidative stress. Progress of oxidation was determined by direct spectrophotometric measurement of conjugated diene formation and by gas chromatographic/mass spectrometric analysis of fatty acids. The oxidative susceptibility of CLA was higher than that of linoleic acid, and comparable to arachidonic acid. When oxidation of PLPC (1.0 mM) was initiated using the lipid-soluble 2,2′-azo*bis*(2,4-dimethylvaleronitrile) or the water-soluble 2,2′-azo*bis*(2-amidinopropane) hydrochloride, the radical scavengers vitamin E and butylated hydroxytoluene (BHT) at 0.75 μM efficiently inhibited PLPC oxidation, as evident from a clear lag phase. In contrast, 0.75 μM CLA did not have any significant effect on PLPC oxidation. Inhibition of PLPC oxidation by higher concentrations of CLA appeared due to competition, not to an antioxidant effect. When oxidation of PLPC was initiated by hydrogen peroxide/Fe^{2+} (500 μM/0.05–20 μM), both vitamin E (1 μM) and ethylene glycol-*bis*(aminoethyl ether) tetraacetic acid (50 μM) efficiently inhibited PLPC oxidation. However, CLA (1–50 μM) did not show a clear protective effect under any of the conditions tested. We conclude that CLA, under these test conditions, does not act as an efficient radical scavenger in any way comparable to vitamin E or BHT. CLA also does not appear to be converted into a metal chelator under metal-ion dependent oxidative stress, as had previously been suggested. On the basis of our observations, a role for CLA as an antioxidant does not seem plausible.

Lipids 30, 599–605 (1995).

"Conjugated linoleic acid" (CLA) is a collective term to describe positional and geometric isomers of linoleic acid (9,12-*cis,cis*-octadecadienoic acid, LA) with conjugated double bonds at carbon atoms 10 and 12 or 9 and 11, and with all possible *cis* and *trans* combinations. Although conjugation of double bonds occurs as part of free radical-mediated oxidation of LA, CLA is a true isomer of LA, in that it does not possess an additional oxygen. Low concentrations of CLA can be found in human blood and tissues (1–6). Its presence may be accounted for *via* two possible pathways. First, CLA may be produced *in vivo* from free radical-mediated oxidation of LA (1,6). Hydrogen abstraction by a free radical species and subsequent diene conjugation produces an LA lipid radical with conjugated diene structure. In the presence of protein, the LA radical may react with protein instead of molecular oxygen, giving rise to a CLA molecule and a protein radical. Second, CLA in human tissues may be derived from dietary sources, as certain foodstuffs, such as fried ground beef and dairy products, have been shown to contain significant amounts of CLA (7–10).

There is a large and growing body of evidence indicating that free radicals and radical-mediated oxidation processes play a role in many pathological conditions, including cancer and atherosclerosis. Thus, considerable effort is invested in the search for natural and synthetic antioxidants that may help prevent or treat these disease states. CLA has been identified as a potent anticarcinogen in a number of model systems (8,11–16), and antioxidant activity has been offered as a possible explanation for its anticarcinogenic effect (11,13,17). Moreover, it also has been suggested that antioxidant properties of CLA may be involved in the observed reduction in atherosclerosis in CLA-fed rabbits (18). However, the information available to date suggests antioxidant activity of CLA is not unambiguous. One study reported that CLA acted as an antioxidant more powerful than α-tocopherol and comparable to butylated hydroxytoluene (BHT) (11). This study, however, was performed using free fatty acids in a phosphate buffer/ethanol mixture (60:40, vol/vol), which is obviously very dissimilar to biological systems. Another study reported antioxidant activity of CLA in rats *in vivo* (13). Unfortunately, the only measure of oxidative damage employed was the thiobarbituric acid-reactive substances assay, a method

*To whom correspondence should be addressed at Nutricia Research, P.O. Box 1, 2700 MA Zoetermeer, The Netherlands.

Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane) hydrochloride; AMVN, 2,2′-azo*bis*(2,4-dimethylvaleronitrile); BHT, butylated hydroxytoluene; CLA, conjugated linoleic acid; GC/MS, gas chromatography/mass spectrometry; LA, linoleic acid; LDL, low-density lipoproteins; PLPC, 1-palmitoyl-2-linoleoyl phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine.

Copyright © 1995 by AOCS Press

known to be indirect and not specific. In addition, CLA antioxidant activity in this study was observed in mammary gland tissue, but not in liver. Also, CLA antioxidant activity was independent of its concentration, and tenfold lower concentrations of vitamin E and butylated hydroxyanisole had a more pronounced effect.

It thus appears that insufficient experimental evidence has been accumulated to substantiate the descriptions of CLA as an *in vitro* and *in vivo* antioxidant. In view of the purported importance of antioxidant activity of CLA in its reported anticarcinogenic and antiatherogenic effects, we considered it essential to reinvestigate CLA's antioxidant potential using defined model systems and more precise analytical methodology. The present studies were undertaken to assess the antioxidant potential of CLA in a model system with phospholipid membranes composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC). These membranes were subjected to various types of metal ion-dependent, as well as metal ion-independent oxidative stress, and the effect of CLA was compared with that of vitamin E, BHT, and EGTA.

MATERIALS AND METHODS

Chemicals. 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and PLPC were obtained from Avanti Polar Lipids (Alabaster, AL). 2,2′-Azo*bis*(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azo*bis*(2-amidinopropane) hydrochloride (AAPH) were from Polysciences (Warrington, PA). BHT and dl-α-tocopherol were purchased from Sigma (St. Louis, MO). Hydrogen peroxide, ferrous ammonium sulfate, and cupric sulfate were obtained from Fisher (San Francisco, CA). CLA was a kind gift of Dr. J. Scimeca at Kraft General Foods (Glenview, IL). CLA was a mixture of isomers with the following composition: 9,11 *(cis,trans)* and 9,11 *(trans,cis)* CLA, 42.2%; 10,12 *(trans,cis)* CLA, 44.2%; 9,11 *(cis,cis)* CLA, 2.2%; 10,12 *(cis,cis)* CLA, 1.4%; 9,11 *(trans,trans)* and 10,12 *(trans,trans)* CLA, 2.3%; 9,12 *(cis,cis)* LA, 4.3%; unidentified, 3.4%. Pure 9,11 *(trans,trans)* CLA and margaric acid (17:0) were obtained from Matreya (Pleasant Gap, PA). CLA stock solutions were made up in ethanol and stored under argon at −20°C. To check the CLA concentration, an absorption spectrum was recorded from 200–300 nm after appropriate dilution, and the CLA concentration was calculated using an average molar extinction coefficient of 27,500 for the absorption maximum at 230–234 nm. All solvents used were of high-performance liquid chromatography grade, and chemicals used were of the highest purity available.

Oxidation of LA and CLA in air. For separate incubations of LA and CLA, a dichloromethane/methanol (1:1) solution containing 0.5 mg LA plus 0.7 mg margaric acid (17:0), or 0.5 mg CLA (mixed isomers) plus 0.7 mg 17:0, was dried down in a roundbottom flask on a rotary evaporator. For incubation of a mixture of LA and CLA together, the solution contained 0.25 mg LA, 0.25 mg CLA, and 0.7 mg 17:0. The dried lipid film in the flask was exposed to air at room temperature. At certain time points, the lipid was dissolved in 2 mL methanol, and duplicate 50-μL samples were taken for gas chromatography/mass spectrometry (GC/MS) analysis of fatty acids. The remaining incubation mixture in the flask was dried down again and further exposed to air. To the sample, 100 μL 0.1 mM BHT was added to stop the oxidation. The methanol was evaporated under nitrogen, and fatty acids were derivatized to the corresponding trimethylsilyl esters by adding 50 μL acetonitrile, 100 μL *bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (Pierce, Rockford, IL), and incubating at 50°C for 20 min. GC/MS analysis was then performed on a Hewlett-Packard (Palo Alto, CA) 5890 Gas Chromatograph coupled to a Hewlett-Packard 5971 Mass Selective Detector under analytical conditions as described before (19) with the exception of a slightly modified temperature program. The initial oven temperature of 50°C was retained for 2 min after injection, then increased to 160°C at a rate of 30°C/min, and subsequently increased to 320°C at a rate of 4°C/min. Loss of LA and CLA due to oxidation was calculated relative to 17:0 (nonoxidizable internal standard).

Metal-independent oxidation of PLPC membranes and effect of antioxidants. The antioxidant activities of CLA, vitamin E (α-tocopherol), and BHT were tested using unsaturated phospholipid model membranes (PLPC vesicles) that were exposed to a steady flux of oxyradicals generated in the lipid phase (AMVN) or in the water phase (AAPH). Unilamellar PLPC vesicles were produced by the ethanol-injection technique (20) as follows. PLPC (2.0 μmol), the appropriate amounts of CLA (mixed isomers), dl-α-tocopherol, or BHT, and AMVN (1.0 μmol) were mixed from stock solutions before each experiment, dried down under nitrogen, and redissolved in a total volume of 70 μL ethanol. This was injected into 2 mL of buffer (10 mM Tris, 150 mM NaCl, pH 7.4, containing 25 μM EGTA) at 40°C in a quartz cuvette with magnetic stirring. When AAPH was used as oxidant, it was not included in the lipid mixture in ethanol, but added to the vesicle suspension from a fresh and cold stock solution in buffer. After 2 min equilibration, the absorbance at 233 nm was zeroed using the complete mixture as its own reference, and absorbance was subsequently monitored in time using a Milton-Roy (Rochester, NY) Spectronic 3000 diode array spectrophotometer. Measurement of absorbance at 233 nm is a direct and sensitive indicator of conjugated diene formation due to oxidation of PLPC substrate, and is frequently used to monitor lipoprotein and model membrane oxidation (21–23). Under the present conditions, an absorbance reading at 233 nm of 0.1 translates into a conjugated diene concentration of approximately 4 μM (using $\varepsilon = 25{,}000\ M^{-1}cm^{-1}$), or an extent of bulk lipid oxidation (1 mM PLPC) of only 0.4%. In some control experiments, POPC vesicles were used instead of PLPC. When fatty acid analysis was called for, samples were taken and processed as described below.

Metal-dependent oxidation of PLPC membranes and effect of antioxidants. PLPC vesicles were prepared by ethanol injection as above. The buffer used in these experiments was 0.5 M sodium phosphate, pH 8.0, and incubations were carried out in glass tubes at room temperature. Hydrogen perox-

ide and Fe^{2+} (ferrous ammonium sulfate) were added from fresh stock solutions at the indicated time points. Samples were taken at specific time points for measurement of 233 nm absorbance (formation of conjugated dienes) using buffer as a blank, and for fatty acid analysis as described below.

Sample processing for fatty acid analysis. At chosen time points during the oxidation of PLPC or POPC vesicles, duplicate 200 µL samples were taken for GC/MS analysis of fatty acids. To stop oxidation, 10 nmol BHT was added to the samples, and 17:0 plus 9,11*(trans,trans)* CLA, a CLA isomer that is virtually absent in the CLA isomer mixture used to test antioxidant activity, were added as internal standards. Samples were processed on ice as much as possible. After adding 1.7 mL deionized water and 100 µL 80 mM HCl, samples were vortex-mixed and extracted three times with 2 mL ethyl acetate. The combined extracts were dried down in a screw-capped tube under nitrogen. In order to prepare fatty acid methyl esters, 200 µL dichloromethane and 800 µL anhydrous methanolic HCl (2 M) was added, the tubes were sealed under nitrogen and left at room temperature for 18 h. Although methylation of fatty acids with methanolic HCl can be achieved much more rapidly when heating the sample (e.g., 85°C for 1 h), we found that the combination of acid and heat results in changes in the CLA isomer distribution pattern, as also found by others (Dr. E.A. Decker, private communication). Methylation with methanolic HCl at room temperature was found to preserve the isomer distribution of the CLA isomer mixture and to esterify free fatty acids as well as glyceride-bound fatty acids.

Finally, 1 mL of water was added to the samples, and fatty acid methyl esters were extracted three times with 2 mL hexane. The pooled hexane extracts were dried down to approximately 100 µL and analyzed by GC/MS as described above. In order to accurately quantify the very small amounts of CLA present in some samples, the mass spectrometer was operated in selected ion monitoring mode, and the molecular ion at *m/z* 294 was used to quantify CLA.

RESULTS AND DISCUSSION

To test whether CLA may act as an antioxidant, two possible antioxidant modes of action for CLA were considered. Our experiments addressed (i) whether CLA could act as a radical scavenging antioxidant, such as vitamin E, or (ii) whether CLA is converted into an antioxidant metal chelator, such as EGTA, under oxidative stress. Oxidative modification of CLA into a metal chelator that subsequently stops radical generation has been suggested to explain the potent antioxidant activity of CLA observed in a previous study (11). For either mode of action, it is important for CLA to be substantially more reactive toward radicals than the substrate it is to protect. Obviously, a radical scavenger should be able to act as a radical sink in order to protect the substrate from radical attack. Likewise, the putative metal chelator formed upon oxidation of CLA should be formed rapidly, before extensive oxidative damage to the substrate has occurred. In view of the proposed relationship between oxidative modification of low-density lipoproteins (LDL) and atherogenesis, we used LA as oxidizable substrate in our experiments, either as free acid or esterified in phospholipid. LA is a major polyunsaturated fatty acid in many tissues, and the major oxidizable fatty acid in LDL.

To assess the oxidative susceptibility of CLA relative to LA, each fatty acid individually [in the presence of margaric acid (17:0) as a nonoxidizable internal standard] was exposed to air. The progress in oxidation was measured by GC analysis of fatty acids after sampling and derivatization as described in the Materials and Methods section. Figure 1A shows that CLA

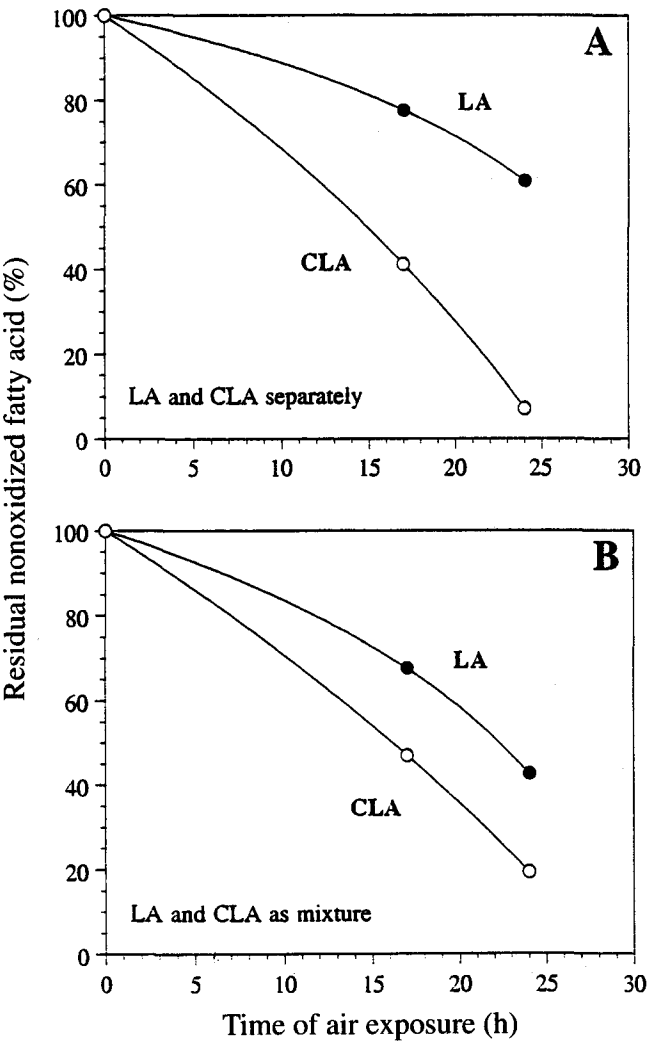


FIG. 1. Oxidation of linoleic acid (LA) (●) and conjugated linoleic acid (CLA) (○) in air when incubated separately (A) and together (B). Lipid in organic solution was dried down in a roundbottom flask on a rotary evaporator and subsequently exposed to air at room temperature. Samples were taken at various time points and the extent of fatty acid substrate loss due to oxidation was determined by gas chromatography/mass spectrometry using 17:0 as a nonoxidizable internal standard. Data points are the mean of duplicate samples. The data spread for each point was within the size of the symbol. The CLA results represent the combined values for individual isomers present in the isomer mixture. Oxidation rates for individual CLA isomers were found to be virtually identical.

was indeed oxidized considerably faster than LA when incubated separately. When exposing a mixture of CLA, LA, and 17:0 to air (Fig. 1B), the order of oxidative susceptibilities was the same, but there was a significant difference. Compared with the separate CLA and LA incubations, the oxidation rate of LA in the CLA/LA mixture had increased, whereas the oxidation rate of CLA had decreased. Additional experiments in which a mixture of LA, CLA, and arachidonic acid (20:4) was present as oxidizable substrate revealed that the oxidative susceptibility of CLA is similar to that of 20:4 (results not shown). Thus, it would appear that, although CLA is more susceptible to oxidation than LA, the difference in oxidative susceptibility between CLA and LA is insufficiently large for CLA to be an efficient antioxidant. In fact, the data in Figure 1B suggest that CLA may even act as a prooxidant, at least under these nonphysiological conditions.

These data are in disagreement with previous reports suggesting that air oxidation of CLA proceeds more slowly than air oxidation of LA (8,11). In those studies, however, a peroxide oxidation product was measured to follow the course of the reaction. As the double bonds in CLA and LA have a different configuration, oxidation of these two fatty acids may proceed *via* different mechanisms and/or give different oxidation products. Assaying for a particular oxidation product instead of measuring the remaining nonoxidized substrate thus may give a misleading picture of the oxidation process. Furthermore, one would expect on theoretical grounds that a conjugated double bond system is more susceptible to radical attack than a nonconjugated double-bond system. We have shown this for the oxidative susceptibility of parinaric acid (18:4), which contains four conjugated double bonds, compared with 20:4, which contains four nonconjugated double-bonds (24).

In a more direct measure of antioxidant potential, PLPC membranes were exposed to oxidative stress, and a spectrophotometric assay of diene conjugation was used to assess the effects of incorporated CLA, α-tocopherol, and BHT. Because CLA itself already contains a conjugated diene structure that can potentially interfere with this measurement, control experiments were performed to assess the behavior of the conjugated double bonds of CLA under AMVN- or AAPH-induced oxidative stress. To that end, vesicles composed of POPC (1.0 mM) plus 0.5 mM AMVN were prepared by ethanol injection (see Materials and Methods section). As the oleoyl chain in POPC contains only one double bond, it cannot form a conjugated diene upon oxidation. Figure 2 shows that a very slight decrease in absorbance occurred over the course of 1 h, probably as a result of the decomposition of AMVN. POPC vesicles containing 25 μM CLA show an elevated initial absorbance at 233 nm of approximately 0.6, which is caused by the conjugated diene structure of CLA. After 1 h of AMVN-induced oxidative stress, fatty acid analysis by GC/MS indicated that approximately 35% of the CLA was lost due to oxidation. No significant change in absorbance was observed during that time, indicating that CLA retains its conjugated diene structure upon oxidation in this

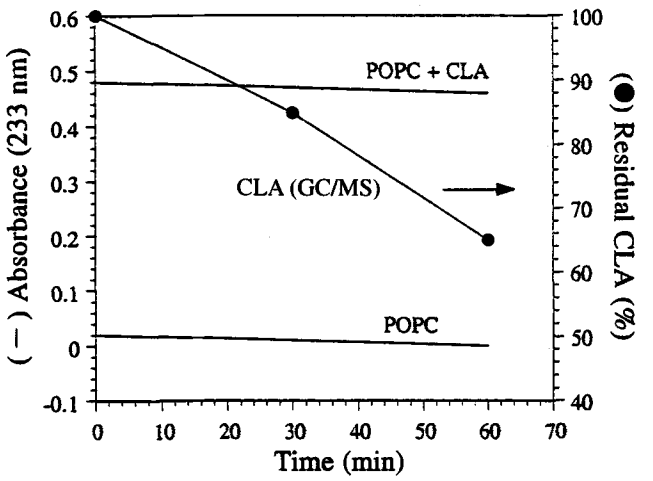


FIG. 2. CLA retains diene conjugation upon 2,2′-azo*bis*(2,4-dimethylvaleronitrile (AMVN)-mediated oxidation. Solid lines: continuous monitoring of absorbance at 233 nm. 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC): control experiment where the absorbance at 233 nm of 1 mM POPC membranes plus 0.5 mM AMVN was monitored in time. POPC + CLA: 1 mM POPC membranes containing 25 μM CLA (mixed isomers) plus 0.5 mM AMVN. (●) Residual nonoxidized CLA determined by gas chromatography/mass spectrometry (GC/MS) analysis of samples taken from the incubation of 1 mM POPC membranes containing 25 μM CLA plus 0.5 mM AMVN. POPC vesicles were prepared by injection of an ethanolic solution containing the appropriate lipid mixture into Tris buffer, pH 7.4. GC/MS data points represent the mean of duplicate determinations. Other abbreviation as in Figure 1.

system. Therefore, the 233 nm absorbance measurements in the PLPC experiments reflect PLPC oxidation, and CLA oxidation or decomposition of AMVN does not interfere with the measurement, to any significant degree.

Oxidation of PLPC vesicles by AMVN-derived radicals resulted in a steady increase in 233 nm absorbance in time; a representative experiment is shown in Figure 3. GC/MS analysis of fatty acids in sample lipid extracts after reduction with sodium borohydride, alkaline methylation, and trimethyl silyl derivatization (25) indicated that the increased absorbance at 233 nm was caused by an isomeric mixture of LA hydroperoxides with the hydroperoxy moiety either in the 9-position (10,12-diene) or in the 13-position (9,11-diene) (results not shown). In the presence of 0.75 μM α-tocopherol, the formation of conjugated dienes was almost completely suppressed during a lag phase, then gradually increased (presumably after α-tocopherol had been consumed). BHT (0.75 μM) appeared more effective than vitamin E in suppressing PLPC oxidation. In contrast, 0.75 μM CLA in the PLPC vesicles did not result in a lag phase in PLPC oxidation. PLPC oxidation after 1 h of oxidative stress in the presence of 0.75 μM CLA was only marginally lower than without CLA. These results indicate that α-tocopherol and BHT are excellent radical-scavenging antioxidants, and that CLA is not effective at all in that respect.

Increasing the concentration of α-tocopherol or BHT present in the vesicles resulted in a proportional increase in the duration of the lag phase in PLPC oxidation (not shown). As

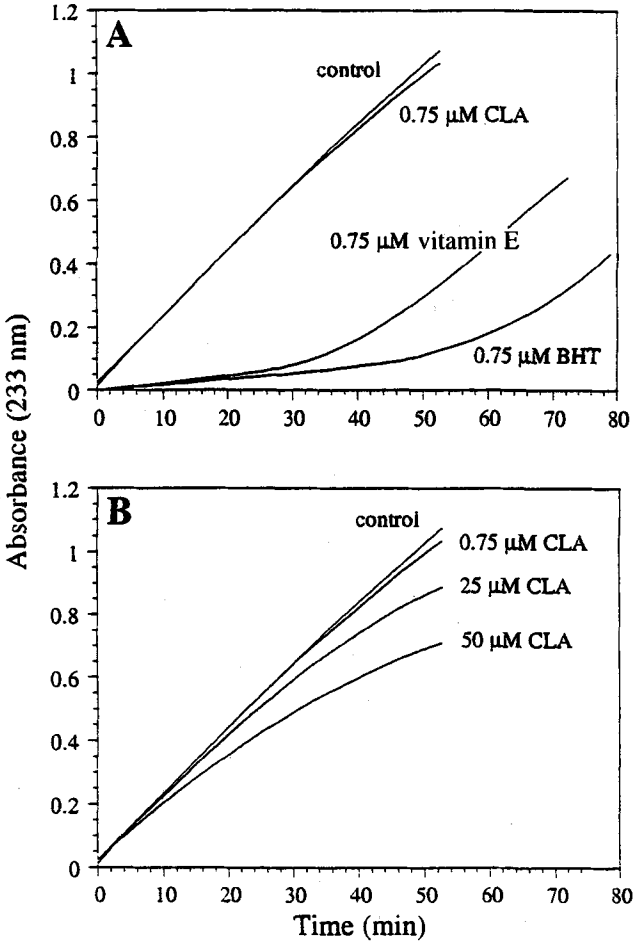


FIG. 3. Effect of CLA, vitamin E, and butylated hydroxytoluene (BHT) on AMVN-mediated oxidation of 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) membranes. (A) Comparative effect of 0.75 μM of CLA (mixed isomers), vitamin E (dl-α-tocopherol), and BHT on PLPC oxidation as monitored by conjugated diene formation. (B) Effect of increasing CLA concentrations on PLPC oxidation. PLPC vesicles (1 mM) were prepared by injection of an ethanolic solution containing the appropriate amounts of PLPC, antioxidant, and AMVN into Tris buffer, pH 7.4. In each experiment, the aborbance at 233 nm was zeroed using the complete mixture as its own reference after allowing 2 min for equilibration. Other abbreviations as in Figures 1 and 2.

shown in Figure 3B, increased concentrations of CLA did proportionally reduce the rate of PLPC oxidation, but a lag phase was not observed at any concentration. GC/MS analysis of fatty acids confirmed that the observed reduction in PLPC oxidation at higher CLA concentrations was due to CLA increasingly competing with LA for available radicals, an effect similar to what could be achieved by adding 20:4 instead of CLA. This would not qualify as a true antioxidant effect unless CLA lipid radicals are not reactive (which they do appear to be, see Fig. 1B), or a specific detoxification mechanism exists for CLA radicals (which is entirely speculative at this point). Generating radicals in the water phase instead of in the lipid phase by using AAPH instead of AMVN yielded very similar results (not shown) and led to the same conclusions as described above.

Subsequent experiments addressed whether CLA could be converted into an antioxidant when exposing PLPC membranes to metal ion-dependent oxidative stress. Figure 4 shows a representative experiment in which oxidative stress was produced by hydrogen peroxide (0.5 mM) plus Fe^{2+} (20 μM). Vitamin E (1 μM) in the vesicle membranes was able to temporarily suppress PLPC oxidation, and 50 μM EGTA prevented lipid oxidation altogether. CLA, however, did not appear to have a protective effect at either 1, 10 (Fig. 4A,B), or 50 μM (not shown). GC/MS data confirmed that CLA was oxidized faster than LA (Fig. 4C), but following the same oxidation pattern as LA. As oxidation of PLPC did not stop or slow down considerably upon oxidation of CLA, apparently CLA was not converted into an antioxidant under these conditions. Many additional experiments were performed in which the reaction conditions were varied. Substituting Tris buffer for phosphate buffer resulted in overall faster oxidation, but did not affect the basic results. Other changes involved variation of the Fe^{2+} concentration from 0.1–20 μM, using Cu^{2+} (0.05–10 μM) instead of Fe^{2+}, using tert-butyl hydroperoxide instead of hydrogen peroxide, and varying CLA concentrations from 1–10 μM. No unequivocal antioxidant effect of CLA could be demonstrated under any of these test conditions, whereas vitamin E and EGTA always exhibited strong antioxidant activity. Therefore, the combination of a peroxide plus transition metal ions, which is a frequent choice of oxidant in model studies and probably a relevant combination *in vivo*, apparently did not give rise to the formation of a potent antioxidant as a result of oxidation of CLA.

In overview, we have not been able to show any clear antioxidant properties of CLA under a variety of experimental conditions. The primary reason why CLA was ineffective appears to be that CLA is insufficiently reactive toward radicals for it to either act as a radical scavenger comparable to vitamin E, or to be rapidly converted into an antioxidant compound as previously proposed (11). The fatty acid oxidation kinetics observed in the present study indicate that CLA behaves similarly to other polyunsaturated fatty acids under oxidative stress. We did not make a detailed study of reaction products formed but, in terms of oxidative susceptibility, CLA was moderately more susceptible than LA, and comparable to 20:4. In a physiological setting, this would mean that, during conversion of CLA into the putative antioxidant chelator (a multi-step process!), significant oxidative damage to LA chains of membrane lipids would likely occur, with even more extensive degradation of arachidonoyl and higher unsaturated fatty acyl chains that are present in biological tissues, including LDL. By the time antioxidant activity of CLA started to have an effect, it would probably be too late.

On a philosophical note, it may even be argued that sequestering transition metal within the lipid phase, as could result from chelation of metal by a CLA oxidation product, could have the opposite of a protective effect. Iron delocalization has been proposed to contribute to increased oxidative stress in a number of pathological conditions, among which sickle cell anemia is a prominent example (26). There-

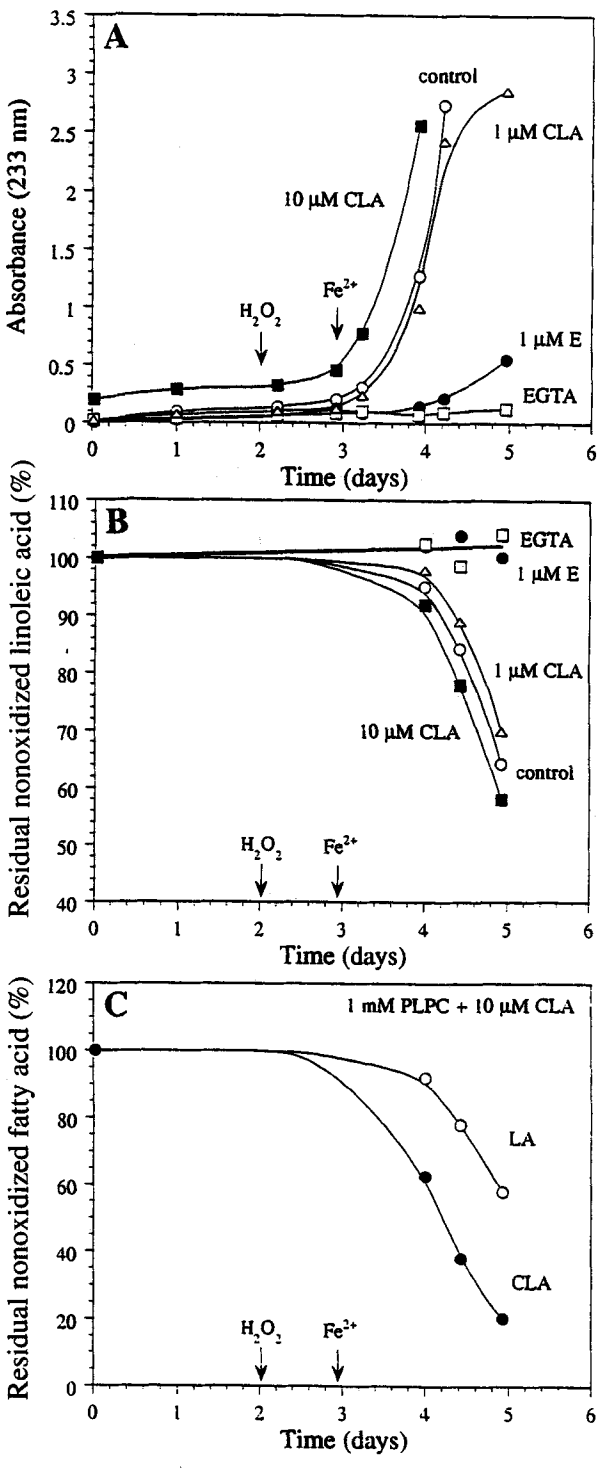


FIG. 4. Effect of CLA, vitamin E, and EGTA on oxidation of PLPC membranes initiated by H_2O_2/Fe^{2+}. (A) Formation of conjugated dienes. (B) Loss of LA as determined by GC/MS analysis of fatty acids in samples taken from the incubation mixture. PLPC vesicles: prepared by ethanol injection of the appropriate lipid mixture in 0.5 M phosphate buffer, pH 8.0. PLPC final concentration was 1.0 mM with 1.0 or 10 μM CLA (mixed isomers) or 1 μM vitamin E where indicated. EGTA (50 μM) was added to buffer before preparation of vesicles. Mixtures were incubated in glass tubes at room temperature. H_2O_2 (500 μM) and Fe^{2+} (20 μM) were added as indicated. (C) Comparison of oxidation of LA and CLA in experiment with 10 μM CLA in 1 mM PLPC vesicles. GC/MS data points represent mean of duplicate determinations. Other abbreviations as in Figures 1 and 3.

fore, unless the metal ion is chelated in a way that also makes it redox-inactive, the binding of metal ion by a CLA oxidation product could potentially be more harmful than protective.

Previously, CLA antioxidant activity had been observed in mammary gland, but not liver tissue of the rat (13). That study employed the widely used, but error-prone thiobarbituric acid-reactive substances assay. Also, CLA antioxidant activity was significant, but not impressive, and did not appear to be concentration-dependent, whereas its anticarcinogenic activity was. The most potent CLA antioxidant activity was observed in the fatty acid model system described in the Introduction section (11), which contained 40% ethanol. Questions may arise with regard to the validity of the observations in that study, since the only assay for assessing oxidation used in those studies also showed CLA to be much less susceptible to oxidation than LA. We have shown here that CLA is in fact more susceptible to oxidation than LA, which confirms the theoretical expectation. Nevertheless, the observation that CLA exhibits antioxidant activity in the buffer/ethanol mixture may have been valid, but the more relevant test conditions employed by us did not demonstrate any antioxidant properties of CLA.

Summarizing, our results clearly indicate that in model membranes CLA does not function as an effective antioxidant or antioxidant precursor.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Joseph Scimeca (Kraft General Foods) for his kind gift of conjugated linoleic acid isomers. This research was supported by a grant from the National Dairy Promotion and Research Board administered in cooperation with the National Dairy Council, and by National Institutes of Health grant DK 34400.

REFERENCES

1. Cawood, P., Wickens, D.G., Iversen, S.A., Braganza, J.M., and Dormandy, T.L. (1983) The Nature of Diene Conjugation in Human Serum, Bile and Duodenal Juice, *FEBS Lett. 162*, 239–243.
2. Fink, R., Marjot, D.H., Cawood, P., Iversen, S.A., Clemens, M.R., Patsalos, P., Norden, A.G., and Dormandy, T.L. (1985) Increased Free-Radical Activity in Alcoholics, *Lancet ii*, 291–294.
3. Harrison, K., Cawood, P., Iversen, A., and Dormandy, T. (1985) Diene Conjugation Patterns in Normal Human Serum, *Life Chem. Rep. 3*, 41–44.
4. Iversen, S.A., Cawood, P., Madigan, M.J., Lawson, A.M., and Dormandy, T.L. (1985) Identification of a Diene Conjugated Component of Human Lipid as Octadeca-9,11-Dienoic Acid, *FEBS Lett. 171*, 320–324.
5. Smith, G.N., Taj, M., and Braganza, J.M. (1991) On the Identification of a Conjugated Diene Component of Duodenal Bile as 9*Z*,11*E*-Octadecadienoic Acid, *Free Radic. Biol. Med. 10*, 13–21.
6. Dormandy, T.L., and Wickens, D.G. (1987) The Experimental and Clinical Pathology of Diene Conjugation, *Chem. Phys. Lipids 45*, 353–364.
7. Parodi, P.W. (1977) Conjugated Octadecadienoic Acids of Milk Fat, *J. Dairy Sci. 60*, 1550–1553.

8. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1987) Anticarcinogens from Fried Ground Beef: Heat-Altered Derivatives of Linoleic Acid, *Carcinogenesis 8*, 1881–1887.
9. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989) Newly Recognized Anticarcinogenic Fatty Acids: Identification and Quantification in Natural and Processed Cheeses. *J. Agric. Food Chem. 37*, 75–81.
10. Britton, M., Fong, C., Wickens, D., and Yudkin, J. (1992) Diet as a Source of Phospholipid Esterified 9,11-Octadecadienoic Acid in Humans, *Clin. Sci. 83*, 97–101.
11. Ha, Y.L., Storkson, J., and Pariza, M.W. (1990) Inhibition of Benzo(*a*)pyrene-Induced Mouse Forestomach Neoplasia by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 50*, 1097–1101.
12. Pariza, M.W. (1991) CLA, A New Cancer Inhibitor in Dairy Products, *Bull. Int. Dairy Fed. 257*, 29–30.
13. Ip, C., Chin, S.F., Scimeca, J.A., and Pariza, M.W. (1991) Mammary Cancer Prevention by Conjugated Dienoic Derivative of Linoleic Acid, *Cancer Res. 51*, 6118–6124.
14. Shultz, T.D., Chew, B.P., Seaman, W.R., and Luedecke, L.O. (1992) Inhibitory Effect of Conjugated Dienoic Derivatives of Linoleic Acid and β-Carotene on the *in vitro* Growth of Human Cancer Cells, *Cancer Lett. 63*, 125–133.
15. Shultz, T.D., Chew, B.P., and Seaman, W.R. (1992) Differential Stimulatory and Inhibitory Responses of Human MCF-7 Breast Cancer Cells to Linoleic Acid and Conjugated Linoleic Acid in Culture, *Anticancer Res. 12*, 2143–2146.
16. Ip, C., Singh, M., Thompson, H.J., and Scimeca, J.A. (1994) Conjugated Linoleic Acid Suppresses Mammary Carcinogenesis and Proliferative Activity of the Mammary Gland in the Rat, *Cancer Res. 54*, 1212–1215.
17. Pariza, M.W., Ha, Y.L., Benjamin, H., Sword, J.T., Grüter, A., Chin, S.F., Storkson, J., Faith, N., and Albright, K. (1991) Formation and Action of Anticarcinogenic Fatty Acids, in *Nutritional and Toxicological Consequences of Food Processing* (Friedman M., ed.) pp. 269–272, Plenum Press, New York.
18. Lee, K.N., Kritchevsky, D., and Pariza, M.W. (1994) Conjugated Linoleic Acid and Atherosclerosis in Rabbits, *Atherosclerosis 108*, 19–25.
19. van den Berg, J.J.M., Winterbourn, C.C., and Kuypers, F.A. (1993) Hypochlorous Acid-Mediated Modification of Cholesterol and Phospholipid: Analysis of Reaction Products by Gas Chromatography-Mass Spectrometry, *J. Lipid Res. 34*, 2005–2012.
20. Batzri, S., and Korn, E.D. (1973) Single Bilayer Liposomes Prepared Without Sonication, *Biochim. Biophys. Acta 298*, 1015–1019.
21. Esterbauer, H., Gebicki, J., Puhl, H., and Jürgens, G. (1992) The Role of Lipid Peroxidation and Antioxidants in Oxidative Modification of LDL, *Free Radic. Biol. Med. 13*, 341–390.
22. Vossen, R.C.R.M., van Dam-Mieras, M.C.E., Hornstra, G., and Zwaal, R.F.A. (1993) Continuous Monitoring of Lipid Peroxidation by Measuring Conjugated Diene Formation in an Aqueous Liposome Suspension, *Lipids 28*, 857–861.
23. Tribble, D.L., van den Berg, J.J.M., Motchnik, P.A., Ames, B.N., Lewis, D.M., Chait, A., and Krauss, R.M. (1994) Oxidative Susceptibility of Low Density Lipoprotein Subfractions Is Related to Their Ubiquinol-10 and α-Tocopherol Content, *Proc. Natl. Acad. Sci. USA 91*, 1183–1187.
24. van den Berg, J.J.M. (1994) Effects of Oxidants and Antioxidants Evaluated Using Parinaric Acid as a Sensitive Probe for Oxidative Stress, *Redox Rep. 1*, 11–21.
25. van den Berg, J.J.M., Op den Kamp, J.A.F., Lubin, B.H., and Kuypers, F.A. (1993) Conformational Changes in Oxidized Phospholipids and Their Preferential Hydrolysis by Phospholipase A_2: A Monolayer Study, *Biochemistry 32*, 4962–4967.
26. Hebbel, R.P. (1991) Beyond Hemoglobin Polymerization: the Red Blood Cell Membrane and Sickle Disease Pathophysiology, *Blood 77*, 214–237.

[Received February 17, 1995; accepted April 12, 1995]

Studies on the Mechanism of the Ursodeoxycholic Acid-Induced Increase in Hepatic Low-Density Lipoprotein Binding

Bernard Bouscarel[a,*], Susan Ceryak[a], Sander J. Robins[b] and Hans Fromm[a]

[a]Division of Gastroenterology and Nutrition, Department of Medicine, The George Washington University Medical Center, Washington, DC 20037 and [b]Lipid Metabolism Laboratory and Department of Medicine, Veterans Administration Medical Center, Boston, Massachusetts 02130

ABSTRACT: Previously, we have shown, in golden Syrian hamsters, that chronic feeding of ursodeoxycholic acid (UDCA), in contrast to that of its 7α-epimer, chenodeoxycholic acid (CDCA), produced a significant increment in hepatic low-density lipoprotein (LDL) uptake, despite similar suppression of bile acid synthesis by both bile acids. Evidence for a direct effect of this bile acid on hepatic LDL metabolism was shown *in vitro*, with isolated hamster hepatocytes, suggesting that this effect was unique to UDCA and specific for receptor-mediated LDL catabolism. The aim of the present study was to define the cellular mechanism(s) associated with this phenomenon, using male golden Syrian hamsters. Regardless of chronic exposure of the liver to either UDCA or CDCA, acute incubation with UDCA consistently resulted in an increase of LDL binding to isolated hepatocytes by 15 to 40%. Furthermore, chronic treatment with either UDCA or CDCA did not result in alterations in lipoprotein particle composition. Likewise, incubation of hepatocytes with UDCA was not associated with a change of the membrane lipid composition. In isolated liver membrane fractions, UDCA increased both the maximum number of LDL binding sites and the affinity constant for LDL by around 35%, suggesting an interaction of UDCA with the LDL receptor, at the plasma membrane level, independent of an effect on receptor cycling. The results of the studies support a role for UDCA in the recruitment of cryptic LDL receptors from a cellular membrane pool, possibly due to the unique localization of UDCA in the plasma membrane lipid bilayer.

Lipids 30, 607–617 (1995).

The low-density lipoprotein (LDL) receptor plays a central role in the regulation of cholesterol homeostasis (1–3). In a number of species, including the hamster, most of receptor-mediated clearance of LDL occurs in the liver (4,5) by rapid internalization of the complex formed by the binding of the apolipoprotein-B_{100}-containing particle to the LDL receptor (6–8). Once in the cell, the LDL particle is delivered to lysosomes, where it is degraded to cholesterol and amino acids, while the LDL receptor generally is cycled back to the membrane (9,10).

The activity of the LDL receptor has been shown to be tightly regulated. Hepatic LDL receptor synthesis and cycling can be markedly suppressed either by dietary cholesterol (3,11,12) or by triglycerides (TGs) ingested together with cholesterol (13,14). Conversely, administration of either cholesterol synthesis inhibitors or bile acid-binding resins increases the number of LDL receptors by increasing the cellular cholesterol demand (15–19). The consequent changes in cellular cholesterol pools modulate the production of LDL receptor mRNA and the synthesis of the LDL receptor (15, 16,20). This indirect adaptive process is slow and requires hours or days.

There is also evidence that LDL receptor activity can be directly regulated independently of the mRNA level. LDL binding to both the human fibroblast and hepatoma cell LDL receptor is altered in the presence of unesterified fatty acids (21,22), as well as ketoconazole (17), an antifungal agent, due to their respective effects on either the plasma membrane lipid composition, or on the LDL receptor conformation. Furthermore, glucagon has also been shown to rapidly increase hepatic LDL receptor activity, possibly by affecting intracellular LDL receptor processing or recycling (23).

Finally, the composition of the LDL particle itself may affect its rate of receptor-mediated catabolism. Denser, smaller LDL subfractions of normal subjects, which were characterized by a higher percentage of protein, as well as a larger cholesterol/TG ratio, exhibited an increased receptor-mediated uptake in cultured fibroblasts when compared to their less dense, larger counterparts (24). In contrast, the receptor-mediated catabolism of LDL, that was isolated from hypertriglyceridemic subjects, was decreased in fibroblasts. This decreased affinity of these LDL particles for the fibroblast LDL receptor was attributed to a TG-induced alteration in the

*To whom correspondence should be addressed at Division of Gastroenterology and Nutrition, Department of Medicine, The George Washington University Medical Center, 2300 I St., N.W., 523 Ross Hall, Washington, DC 20037.

Abbreviations: CDCA, chenodeoxycholic acid; FC, free cholesterol; GUDCA, glycine conjugate of UDCA; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PL, phospholipid; TG, triglyceride; TUDCA, taurine conjugate of UDCA; UDCA, ursodeoxycholic acid; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

tertiary structure of the apolipoprotein, independent of the LDL receptor binding domain (25).

We have previously shown *in vivo*, in the golden Syrian hamster, that chronic feeding of ursodeoxycholic acid (UDCA), in contrast to that of its 7α-epimer, chenodeoxycholic acid (CDCA), evoked a significant increment in hepatic LDL uptake, in spite of both a 55–71% enrichment of the bile acid pool with CDCA and a marked suppression of bile acid synthesis (26). We also have documented this increase of LDL receptor-dependent LDL uptake and degradation *in vitro*, upon acute exposure to UDCA, in hepatocytes isolated from chow-fed hamsters (27). This direct effect of UDCA, which is time- and concentration-dependent, did not occur with either CDCA or lithocholic acid (LCA), and was not associated with a toxic effect of the bile acid.

The present investigation was undertaken in order to elucidate the mechanism of both the short-term (direct) and long-term effect of UDCA on the LDL receptor. The direct effect of UDCA on hepatic LDL binding was studied following chronic exposure to UDCA and CDCA, respectively. The plasma lipoprotein composition was also determined following bile acid feeding, in order to investigate the possibility of LDL particle alteration. Furthermore, to explore the membrane-related effect of UDCA as a mechanism by which UDCA directly enhances hepatic LDL binding, the LDL binding to isolated liver membranes was studied, and the plasma membrane lipid composition was determined in the presence and absence of UDCA.

MATERIALS AND METHODS

Materials. $Na^{125}I$ (specific activity 16–20 mCi/μg) was purchased from Amersham Corporation (Arlington Heights, IL). Gelatin and bovine serum albumin (fraction V) were purchased from Sigma (St. Louis, MO). UDCA was supplied by Tokyo Tanabe Co. (Tokyo, Japan), CDCA by Dr. Falk GmbH and Co. (Freiburg, Germany). UDCA and CDCA were 98–99% pure, as judged by gas–liquid chromatography. All other chemicals used were of analytical or high-performance liquid chromatography grade available from commercial sources.

Animals. Unless otherwise indicated, hamsters were maintained on a standard chow diet. For chronic bile acid treatment studies, three groups of male golden Syrian hamsters (115–130 g body weight; Harlan Sprague Dawley, Indianapolis, IN), consisting of eight animals per group, were fed a standard rodent chow diet (Ralston Purina Co., St. Louis, MO) for four weeks. One group received the diet alone (control), the second received, in addition, 0.1% UDCA, and the third, 0.1% CDCA. There were no differences among the treatment groups as far as food intake and weight gain were concerned. Mean food intake was 7.5 g/animal/day, while mean body weight ranged from 133.1–133.6 g following treatment. Both bile acids were well tolerated by the animals, and the livers appeared macroscopically normal after four weeks of bile acid feeding. Previously we have reported that light microscopic examination of the livers of hamsters fed the same bile acid-supplemented diets showed normal findings (26).

Hepatocyte isolation. Hepatocytes were isolated from male Golden Syrian hamsters by the collagenase perfusion technique of Exton (28), as previously described (27). The cells were suspended at a concentration of approximately 40–50 mg wet wt/mL of Krebs-Henseleit bicarbonate buffer, containing 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 1.5% gelatin, pH 7.4. Prior to each experiment, hepatocytes were incubated for 20–30 min at 37°C under constant agitation and gassing, in order for the cells to reach a steady-state (27).

Separation of plasma lipoprotein fractions. Blood was collected from healthy human subjects, as well as from both bile acid-treated and untreated hamsters, into EDTA-containing vacuum collection tubes. The plasma was separated by centrifugation at 300 × *g* for 40 min at 4°C. Plasma lipoproteins were separated by density gradient ultracentrifugation as previously described (26,27), according to the method of Redgrave *et al.* (29). Layers were aspirated from the centrifuge tubes at the following density ranges, in order to assess the lipoprotein composition following bile acid feeding: <1.007 g/mL, very low density lipoprotein (VLDL); 1.007–1.019 g/mL, intermediate-density lipoprotein (IDL); 1.020–1.064 g/mL, LDL; 1.065–1.210 g/mL, high-density lipoprotein (HDL); >1.210 g/mL, albumin-rich, lipoprotein-free fraction. The LDL that was used in the study of LDL binding to hamster hepatocytes and isolated liver membrane fractions, respectively, was isolated at a density range of 1.020–1.060 g/mL, and was further purified and concentrated following adjustment to a density of 1.063 g/mL, and recentrifugation (27).

Determination of lipoprotein composition. The total protein concentration of the different lipoprotein fractions was determined by the method of Bradford (30), using the Bio-Rad® protein assay (Bio-Rad Laboratories, Richmond, CA). Enzymatic assays were used to measure cholesterol and TG (BioDynamics; Boehringer Mannheim, Indianapolis, IN), as well as choline-containing phospholipid (PL) (Nippon Shoji Kaisha Ltd., Higashi-Ko, Osaka, Japan) concentrations.

The apoprotein composition of the different lipoprotein fractions was assessed by SDS–polyacrylamide gel electrophoresis (PAGE) according to the method of Irwin *et al.* (31), followed by silver staining (32). The relative intensity of the respective apoprotein bands was determined densitometrically with a Hoeffer GS300 scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA). The electrophoretic mobility of the individual lipoprotein fractions on thin agarose gels was determined using the method of Elevitch *et al.* (33), followed by staining with Fat Red 7B (Analytical Chemists, Inc., Palo Alto, CA)(34).

Biliary bile acid composition. In order to confirm the enrichment of the respective bile acid pool by either UDCA or CDCA in the hamsters receiving the bile acid-supplemented diets, the relative bile acid composition was determined by gas–liquid chromatography in gallbladder bile as previously described (26,35).

LDL radiolabelling. Native hamster LDL isolated from untreated animals was labelled with [^{125}I]sodium iodide to a specific radioactivity of 80–200 cpm/ng of protein as previously described (27) using the iodine monochloride technique (36) as modified for lipoproteins (37), and was dialyzed overnight against 0.15 M NaCl, 0.01 M Tris, 0.001 M NaN_3, pH 7.4. ^{125}I-labelled LDL was filtered through a 0.45 μm cellulose nitrate membrane filter (Millipore Corp., Bedford, MA), and used within four days.

Hepatocellular ^{125}I-LDL binding. For a period of three consecutive days, all studies of the acute effect of UDCA on hepatic LDL binding were performed using hepatocytes isolated from one animal of a given chronic treatment group, using the same [^{125}I]LDL preparation. The order of animals studied from each chronic treatment group was varied for each set of experiments.

The binding experiments were performed as previously described (27). Following five washes with ice-cold bicarbonate buffer, the hepatocytes (150–250 mg protein/mL) were incubated at 4°C with increasing concentrations of ^{125}I-labelled hamster LDL (1–90 μg protein/mL) in the presence and the absence of 700 μM UDCA. Previous studies have shown that, at this concentration, UDCA is not toxic to the cell (27,38). After one hour, the cell-associated radioactivity was measured in a Beckman model 4000 gamma-radiation counter (Beckman Instruments, Fullerton, CA). Nonspecific binding was determined by incubating the cells under the same conditions as described, but with an excess of native human LDL (1–2 mg/mL) (27). Specific binding was determined by subtracting nonspecific binding from total binding. Saturation binding curves were derived by plotting the amount of ^{125}I-labelled hamster LDL specifically bound per mg of cell protein on the y axis against the concentration of unbound LDL on the x axis. The maximum number of binding sites (B_{max}, ng/mg protein) and the dissociation constant (K_D, μg LDL protein/mL) were calculated from the saturation curves, as previously described (27).

Liver membrane isolation. Livers obtained from male Golden Syrian hamsters were homogenized with 150 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris-HCL, pH 7.5 using a Dounce homogenizer as previously described (39). Liver membranes were prepared by the method of Kovanen *et al.* (40). All isolation and preparative procedures were carried out at 4°C. The homogenates were centrifuged at 500 × *g* for 5 min. The supernatant was then centrifuged at 8,000 × *g* for 15 min, and the 8,000 × *g* supernatant was centrifuged at 100,000 × *g* for 60 min using a Beckman 70.1 Ti fixed-angle rotor. The resulting pellet was resuspended in the same buffer and recentrifuged at 100,000 × *g* for 60 min. The final pellet was used either immediately, or quickly frozen in liquid N_2, stored at –70°C, and used within two weeks.

Binding of ^{125}I-LDL to isolated liver membrane preparations. Membrane pellets were resuspended in 50 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5 and sonicated for 20 s, using a Sonifier Cell Disrupter (Branson Sonic Power Co., Plainview, NY). The binding of LDL to liver membranes was essentially carried out as described by Kovanen *et al.* (40), in a total volume of 80 μL 100 mM NaCl, 0.5 mM $CaCl_2$, 50 mM Tris-HCL, pH 7.5, containing 20 mg/mL bovine serum albumin. Liver membranes (100 μg protein/mL) were incubated with 1–100 μg protein/mL [^{125}I]-labelled hamster LDL for 60 min at 22°C in the presence and absence of 700 μM UDCA. Binding was rapid, and equilibrium was reached in less than 20 min and remained stable for at least one hour. The nonspecific binding was determined under the same conditions but in the presence of an excess of unlabeled human LDL (1–2 mg/mL). Saturation binding curves were derived, and the data were analyzed according to the method of Scatchard (41) by linear and nonlinear curve fitting, using the EBDA/LIGAND program (Elsevier-BIOSOFT®, Cambridge, United Kingdom), as modified for microcomputers (42). The B_{max} (ng/mg protein) and K_D (μg LDL protein/mL) were calculated from the equation of the regression line.

Assessment of release of cellular lipids into media. Isolated hepatocytes were incubated for either 10 or 120 min at 37°C in bicarbonate buffer containing 1.5% gelatin, and in the presence and absence of 700 μM UDCA. At the end of these incubation periods, the incubation buffer was collected and the cells were washed and centrifuged at 50 × *g* for 3 min at 4°C. Total lipids were extracted from the incubation medium as well from the cells by adding 3.75 mL $CHCl_3$/methanol, 1:2 (vol/vol), for each mL of aqueous suspension according to the method of Bligh and Dyer (43). Lipid extracts were kept under nitrogen in glass tubes to prevent oxidative degradation. PC molecular species and cholesterol amounts were determined by previously published methods (44,45), using several different high-performance liquid chromatography procedures to separate these lipids. Quantitation was performed by integration of peak areas in conjunction with internal standards. Total PL was quantitated by phosphorus measurement (46).

Statistical analysis. The paired *t*-test was used to compare the acute effect of UDCA treatment with that of the respective controls. An analysis of variance was used to compare the differences in the measured parameters as a result of bile acid feeding.

RESULTS

Effect of acute UDCA treatment on specific [^{125}I]-labelled hamster LDL binding at 4°C to hepatocytes isolated from chronic UDCA- or CDCA-fed hamsters. The specific binding of increasing concentrations (1–90 μg protein/mL) of [^{125}I]hamster LDL to hepatocytes isolated from animals fed either a standard chow diet or a 0.1% UDCA- or CDCA-supplemented diet for four weeks was studied at 4°C, in the presence and absence of 700 μM UDCA. Nonspecific binding, which was measured in the presence of an excess of native human LDL, represented 30–40% of the total binding, and was not affected by either acute or chronic bile acid treatment. Saturation of specific binding occurred at LDL concentrations of 30–50 μg/mL. The apparent binding constants, B_{max}, are

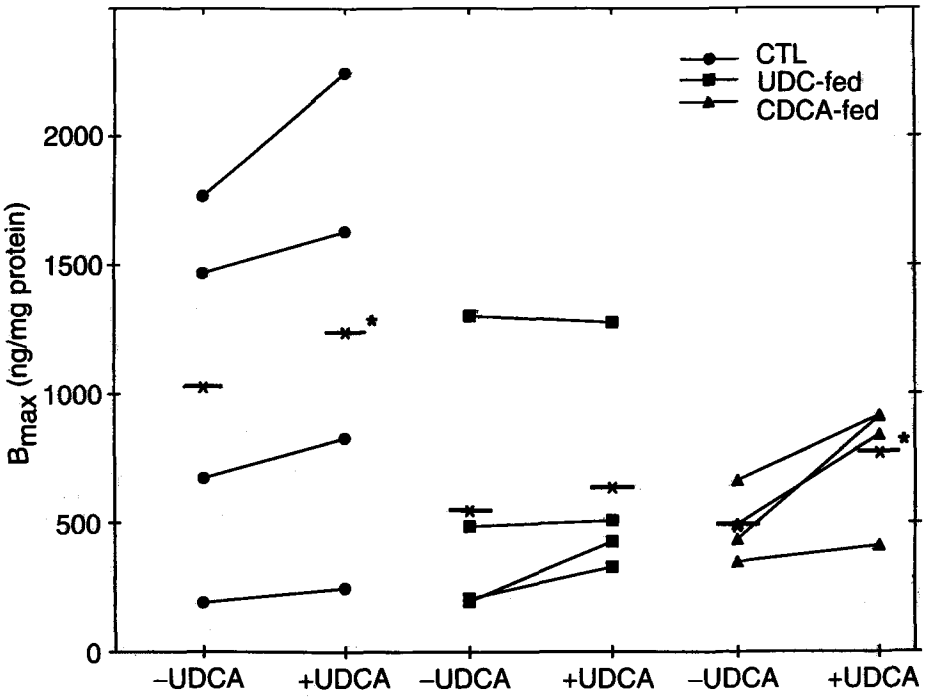


FIG. 1. Effect of acute treatment with ursodeoxycholic acid (UDCA) on the binding of [^{125}I]hamster low-density lipoprotein (LDL) to hepatocytes isolated from hamsters following chronic feeding of either a normal diet, or a normal diet supplemented with either UDCA or chenodeoxycholic acid (CDCA). For each experiment, hepatocytes were isolated from one hamster of each treatment group on three consecutive days, following four weeks of feeding either a normal chow diet alone (control, CTL), or the same diet supplemented with either 0.1% UDCA (UDCA-fed) or CDCA (CDCA-fed). Hepatocytes (150–200 μg protein/mL) were incubated at 4°C for 60 min with 1–90 μg/mL of [^{125}I]hamster LDL protein, and specific LDL binding was determined both in the absence (–UDCA) and presence of 700 μM UDCA (+UDCA). The unbound [^{125}I]hamster LDL was separated from the bound by rapid centrifugation of the cells (2000 × *g* for 2 min) and by washing them three times with ice-cold incubation buffer. Nonspecific binding, which was determined in a parallel set of experiments by incubating the cells under the same conditions with an excess of unlabelled native human LDL (1–2 mg/mL), was subtracted from the total binding. The respective apparent binding constant, B_{max}, was derived from analysis of the saturation curves. Horizontal bars represent the means of four experiments. *Significantly different from respective control incubation, $P < 0.05$.

shown in Figure 1, and were derived from analysis of the respective saturation curves.

As shown previously (27), when hepatocytes isolated from hamsters fed a standard chow diet were incubated with 700 μM UDCA, the maximum LDL binding (B_{max}) significantly increased by approximately 20%, from a control value of 1024 ± 361 to 1238 ± 439 ng/mg protein (Fig. 1).

The apparent decrease in LDL binding as a result of UDCA feeding was not statistically significant (Fig. 1). Furthermore, the B_{max} of 547 ± 261 ng/mg protein, determined in hepatocytes isolated from UDCA-fed animals, was not significantly altered after incubation of the cells with 700 μM UDCA (634 ± 218 ng/mg protein).

The tendency for the four-week treatment with CDCA to attenuate the maximum LDL bound to the hepatocytes was not significant, compared to the respective control-fed animals. However, the B_{max} value of 769 ± 241 ng/mg protein, after the acute exposure to 700 μM UDCA, was significantly higher than that of 483 ± 134 ng/mg protein in the control experiment (Fig. 1).

The K_D values were not significantly altered by either acute incubation with 700 μM UDCA, or by bile acid feeding, and ranged from 14.6 to 25.9 μg LDL protein/mL, under control conditions, and from 12.4 to 26.6 μg LDL protein/mL following one hour incubation with 700 μM UDCA (data not shown).

Effect of chronic UDCA and CDCA treatment on hamster plasma lipoprotein composition. Previously it has been shown that the relative abundance of components of the LDL particle could affect its receptor-mediated catabolism (24,25). To determine whether or not chronic feeding of UDCA or CDCA could alter the lipoprotein composition, and thus offer a possible mechanism for their observed effects on hepatic LDL uptake *in vivo* (26), the plasma lipoprotein composition was analyzed in hamsters after four weeks of feeding UDCA, CDCA, or the control diet.

The relative molar percent composition of protein, cholesterol, PL, and TG in the LDL- and HDL-containing fractions are shown in Tables 1 and 2, respectively. Neither the UDCA nor CDCA feeding resulted in any significant modification of

TABLE 1
Biliary Bile Acid Composition in Hamsters Following Chronic Bile Acid Feeding[a]

Treatment	LCA (%)	DCA (%)	CDCA (%)	UDCA (%)	CA (%)	Other[b] (%)
Control	1.2 ± 0.3	9.3 ± 1.4	28.7 ± 2.6	1.0 ± 0.1	54.0 ± 3.7	5.6 ± 2.0
UDCA	3.4 ± 0.4	3.9 ± 0.6*	49.4 ± 1.7*	20.6 ± 1.2*	16.8 ± 1.6*	6.0 ± 0.6
CDCA	2.9 ± 0.4	2.5 ± 0.1*	74.1 ± 2.2*	n.d.	13.4 ± 1.9*	7.0 ± 1.3

[a]Relative bile acid composition was determined by gas–liquid chromatography in gallbladder bile obtained from hamsters following four weeks of feeding either a normal chow diet alone (control), or the same diet supplemented with either 0.1% urosdeoxycholic acid (UDCA) or 0.1% chenodeoxycholic acid (CDCA). Results are the means ± SEM of determinations in four animals of each treatment group. *Significantly different in comparison to control-fed animals, $P < 0.05$. LCA, lithocholic acid; DCA, deoxycholic acid; CA, cholic acid; n.d., not detected.
[b]Other, unidentified gas chromatographic peaks.

TABLE 2
Relative Plasma LDL Composition Following Chronic Administration of the UDCA- and the CDCA-Supplemented Diets[a]

	% Composition by weight			
Treatment	Protein	Cholesterol	Choline-containing phospholipid	Triglyceride
Control	11.2 ± 1.8	23.9 ± 3.1	49.2 ± 2.2	15.7 ± 1.0
UDCA	15.0 ± 3.3	24.4 ± 3.1	40.3 ± 5.6	16.6 ± 1.4
CDCA	11.8 ± 4.9	34.1 ± 5.2	38.5 ± 4.0	15.5 ± 1.9

[a]Low-density lipoprotein (LDL) were isolated by density gradient ultracentrifugation from plasma obtained from hamsters following four weeks of feeding either a normal chow diet alone (control), or the same diet supplemented with either 0.1% UDCA or 0.1% CDCA. Relative plasma lipoprotein protein, cholesterol, phospholipid, and triglyceride composition was determined by colorimetric methods. Results are the means ± SEM of determinations in four animals of each treatment group. See Table 1 for other abbreviations.

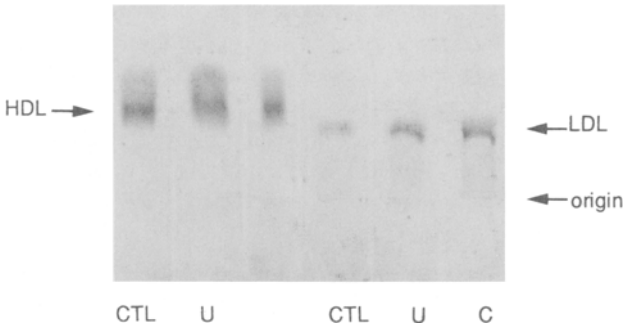


FIG. 2. Agarose gel electrophoretic mobility of LDL and high-density lipoprotein (HDL) fractions isolated from plasma obtained from hamsters following chronic feeding of UDCA and CDCA, respectively. LDL and HDL were isolated by density gradient ultracentrifugation from plasma obtained from hamsters following four weeks of feeding either a normal chow diet alone (control, CTL), or the same diet supplemented with 0.1% UDCA (U) and CDCA (C), respectively. Following electrophoresis on thin agarose gels, the lipoproteins were visualized by lipid staining with a solution of Fat Red 7B (Analytical Chemists, Inc., Palo Alto, CA). See Figure 1 for other abbreviations.

the plasma lipoprotein composition as far as total protein, cholesterol, PL, or TG was concerned. However, there was an increase, although statistically not significant, in the relative proportion of LDL cholesterol from 23.9 ± 3.1% in the controls to 34.1 ± 5.2% in the CDCA-fed animals. Furthermore, no effect of bile acid feeding was observed in the relative composition of VLDL- or IDL-containing fractions (data not shown).

The absence of alterations in the protein composition was further confirmed by SDS–PAGE of the delipidated lipoprotein fractions. This revealed no apparent differences in either the protein composition or mobility in the three experimental groups. Apolipoprotein B_{100} comprised 95.5 ± 4.5, 93.8 ± 6.2, and 93.5 ± 6.5% of the LDL proteins in LDL isolated from control, UDCA-, and CDCA-fed animals, respectively. The variable presence of proteins at an apparent M_r of 36 kDa (3–4%) and 25 kDa (1–2%) probably represented contamination of apolipoprotein E and AI, respectively, from the HDL fraction. HDL isolated from the control, UDCA-, and CDCA-treated hamsters was characterized by a respective apolipoprotein AI component representing 94.8 ± 2.4, 95.6 ± 1.4, and 95.7 ± 0.3% of the total proteins. Protein bands were also detected at an apparent M_r of 64 kDa (<1%), 48 kDa (1–2%), 36 kDa (2–3%), 13 kDa (<1%), and 12 kDa (<1%), corresponding to the respective molecular weights for albumin, and apolipoproteins AIV, E, and Cs.

In addition, the apparent electrophoretic mobility of the lipoprotein fractions on agarose gels also was not altered by bile acid feeding. As shown in Figure 2, similar β and α migration patterns were obtained with the respective LDL and HDL fractions isolated from the plasma of animals following chronic bile acid treatment.

Effect of chronic UDCA and CDCA treatment on hamster biliary bile acid composition. As shown in Table 3, the

TABLE 3
Relative Plasma High-Density Lipoprotein Composition Following Chronic Administration of the UDCA- and the CDCA-Supplemented Diets[a]

	% Composition by weight			
Treatment	Protein	Cholesterol	Choline-containing phospholipid	Triglyceride
Control	40.5 ± 2.6	16.4 ± 0.7	40.1 ± 2.6	2.9 ± 0.6
UDCA	42.2 ± 1.4	16.5 ± 0.8	36.2 ± 1.6	5.1 ± 1.3
CDCA	41.5 ± 1.4	19.2 ± 2.7	35.4 ± 2.0	3.8 ± 0.6

[a]For experimental details and abbreviations, see legends of Tables 1 and 2.

CDCA content in bile increased significantly during chronic treatment with both CDCA and UDCA, as has been previously observed in this model, due to the intestinal biotransformation of UDCA to CDCA (26,47). While significantly increased compared to control and CDCA-treated animals, the 20.6% enrichment of bile in UDCA in the UDCA-treated animals was considerably less than that observed in humans under comparable conditions (48). The relative percentages of biliary deoxycholic and cholic acids decreased significantly due to the relative enrichment in both the respective fed bile acid, and its metabolites.

Effect of UDCA on specific ^{125}I-labelled LDL binding to membranes isolated from hamster liver. From our previous studies (27), we concluded that the effect of UDCA was specific to the LDL receptor, since it was abolished following proteolytic degradation of the LDL receptor. One hypothesis proposed was that UDCA may uncover, or make accessible, LDL receptors, which are sequestered in the plasma membrane. Therefore, the effect of 700 μM UDCA on the binding of LDL was determined using isolated liver membrane fractions. Nonspecific binding increased linearly with LDL concentration, and represented 25–35% of the total LDL binding. The results of the specific binding studies are shown in Table 4. Scatchard analysis of the saturation curve showed that UDCA increased the B_{max} by 32% (Fig. 3 and Table 4). This UDCA-induced increase in LDL binding is similar to that previously observed in isolated hamster hepatocytes (27). However, in contrast to observations in intact hepatocytes, UDCA caused a significant decrease in the binding affinity by 37% (Table 4). The Hill coefficient was around 0.98 in

TABLE 4
Effect of UDCA on LDL Receptor Number and Affinity in Hepatic Membrane Fractions[a]

	K_D (μg LDL protein/mL)	B_{max} (ng/mg of protein)
–UDCA	67.4 ± 7.4	1338 ± 210
+UDCA	92.3 ± 13.9*	1763 ± 251**

[a]The binding of increasing concentrations (1–100 μg protein/mL) of [^{125}I]hamster LDL to isolated hamster liver membrane fractions (100 μg protein/mL) was measured at 22°C in the presence and absence of 700 μM UDCA, in a total volume of 80 μL. Nonspecific binding was determined by incubating the membrane fractions under the same conditions but in the presence of an excess of native human LDL (1–2 mg/mL). The unbound LDL was separated from the bound by centrifugation at 100,000 × *g* for 5 min. The apparent binding constants were determined by Scatchard analysis of the saturation binding curves, as represented in Figure 3. The results are the mean ± SEM of five different experiments. *$P < 0.05$; **$P < 0.02$—significantly different in the presence of UDCA. B_{max}, maximum number of binding sites; K_D, affinity constant. See Table 1 for other abbreviations.

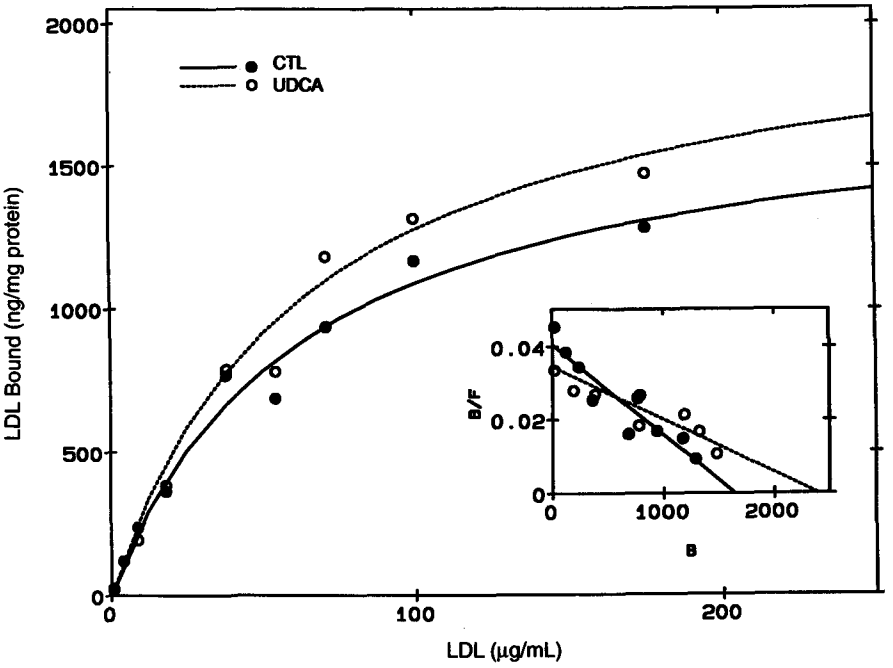


FIG. 3. Effect of UDCA on the binding of [^{125}I]hamster LDL to membranes isolated from hamster liver. For experimental details, see legend of Table 4, and the Materials and Methods section. Hamster liver membrane vesicles were incubated at 22°C with increasing concentrations (1–100 μg protein/mL) of [^{125}I]hamster LDL, in the presence and absence of UDCA. The data were analyzed according to the method of Scatchard by linear and nonlinear curve fitting, using the EBDA/LIGAND program (Elsevier-BIOSOFT®, Cambridge, United Kingdom), as modified for microcomputers. The saturation curve and Scatchard plot (inset) of the specific LDL binding is representative of five experiments. In this experiment, the Hill coefficient was 0.960 and 0.971, respectively, in the presence and absence of UDCA, suggesting the presence of a single homogenous population of LDL binding sites, while the corresponding coefficient of correlation was –0.903 and –0.933. See Figure 1 for abbreviations. B/F, bond/free.

TABLE 5
Hepatocellular Lipid Composition[a]

	Cells[b]				Media			
	Control	+UDCA	Control	+UDCA	Control	+UDCA	Control	+UDCA
	10 min		120 min		10 min		120 min	
PL (mM)	1.76	1.73	1.60	1.51	0.09	0.09	0.18	0.17
PC (mM)	0.80	0.85	0.73	0.74	0.04	0.04	0.08	0.08
FC (mM)	0.17	0.17	0.16	0.15	0.02	0.02	0.03	0.03
%PL as PC	45.7	49.0	45.9	49.1	47.8	47.4	43.8	48.9
FC/PC	0.22	0.20	0.22	0.20	0.47	0.47	0.44	0.42

[a]The cell-associated lipids (Cells) and the lipids released into the incubation media (Media) were extracted into chloroform/methanol (1:2, vol/vol) and measured as indicated in Materials and Methods, following incubation of isolated hamster hepatocytes at 37°C for 10 and 120 min, respectively, in the presence and absence of 700 μM UDCA. The results represent one determination. PL, total phospholipids; PC, phosphatidylcholine; FC, free cholesterol. See Table 1 for other abbreviation.
[b]A 1 mL cell suspension contains approximately 45 mg/mL cells, and 1 mg of cells contains 0.13 ± 0.009 mg protein.

both the absence and presence of UDCA, suggesting the presence of a single homogeneous population of binding sites.

The possibility was considered that perfusion of the liver with collagenase, as it is performed for the isolation of hepatocytes, could decrease the reactivity of the LDL receptors. UDCA may counteract the collagenase effect by stabilizing the plasma membrane, with consequent normalization of LDL binding. To assess the possibility of a damaging effect of collagenase, the binding of 70 μg/mL LDL was measured using liver membranes incubated without or with 0.4 mg/mL collagenase for 60 min at 4°C. The concentration of collagenase selected corresponded to five times the concentration used in the liver perfusion. The LDL specifically bound (1210.8 ± 32.7 ng/mg protein, n = 3), was not significantly changed after incubation of the membranes with collagenase (1279.5 ± 30.2 ng/mg protein, n = 3).

Effect of UDCA on hepatocellular lipid composition. As amphipathic molecules, bile acids are known to have detergent action. Therefore, one of the possible mechanisms by which UDCA could induce this increment in hepatic LDL binding and uptake is to alter the plasma membrane lipid composition, and, consequently, affect the conformation of the LDL receptor in the membrane lipid bilayer. Therefore, the hepatocellular lipid composition following 10 and 120 min incubation of the hepatocytes at 37°C was determined in the presence and absence of 700 μM UDCA (Table 5). The cellular concentrations of total PL, total PC, and free cholesterol (FC) in the 10-min control incubation were approximately 1.8, 0.8, and 0.2 mM, respectively. The corresponding values in the UDCA incubation were almost identical. Similar observations were made in the 120-min incubation experiments, which showed no differences in the cellular lipid concentrations between the control and UDCA incubations.

Incubation with UDCA also did not induce any changes in the release of cellular lipids into the incubation media at either 10 or 120 min (Table 5). However, in both the control and UDCA incubations the total PL concentration in the media was 0.09 and 0.09 mM, respectively, at 10 min and 0.18 and 0.17 mM at 120 min. The respective values for the total PC concentration were 0.04 and 0.04 mM, at 10 min, and 0.08 and 0.08 mM, at 120 min. FC showed barely an increase from 10 to 120 min of incubation, i.e., from 0.02 and 0.02 mM, respectively, to 0.03 and 0.03 mM.

When the PC content was expressed as % of phospholipids or when the ratio FC/PC was calculated, there was no change during the 120-min cell incubation at 37°C either in the presence or absence of UDCA (Table 5). Furthermore, when the different PC molecular species were determined in cells and in incubation buffer, the relative fatty acid composition of PC was not altered in the period of incubation, during which the effect of UDCA was studied (Table 6).

DISCUSSION

The results of the present studies are in keeping with previous reports of the UDCA-induced enhancement of hepatic LDL catabolism (26,27). Regardless of chronic exposure of the liver to either UDCA or CDCA, acute incubation with UDCA for one hour consistently increased the B_{max} for LDL binding to isolated hamster hepatocytes, without altering the binding affinity.

Malavolti *et al.* (26) observed that the unique UDCA-induced enhancement of hepatic LDL uptake *in vivo* occurred in spite of suppression of bile acid synthesis. The extensive biotransformation of UDCA to CDCA in the hamster was thought to have been the probable cause for the observed suppression of bile acid synthesis, since, in contrast to UCDA, CDCA is a potent inhibitor of bile acid synthesis (49,50). Because chronic suppression of bile acid synthesis has been associated with LDL receptor downregulation (51), a direct effect of UDCA was postulated to account for the increment in hepatic LDL uptake *in vivo*. Indeed, a direct effect of this bile acid on hepatocellular LDL metabolism was later demonstrated *in vitro* (27). The results of the present study confirm the existence of a direct effect of UDCA *in vivo*. The tendency for chronic feeding of either UDCA or CDCA to decrease specific LDL binding to isolated hepatocytes could be due to the downregulation of LDL receptor synthesis by a

TABLE 6
Phosphatidylcholine Molecular Species[a]

	Cells[b] (molar %)				Media (molar %)			
	Control	+UDCA	Control	+UDCA	Control	+UDCA	Control	+UDCA
	10 min		120 min		10 min		120 min	
16:0-22:6	12.0	12.1	12.3	11.8	9.8	10.1	8.7	9.7
20:4	6.9	7.1	7.2	7.9	7.4	6.5	7.2	6.4
18:2	19.8	19.2	19.9	19.4	19.8	20.2	21.6	20.7
18:1	15.2	14.8	15.2	14.8	17.7	16.7	18.7	17.2
18:0-22:6	5.6	5.6	5.5	5.1	4.4	5.5	3.6	5.4
20:4	5.9	5.9	6.1	6.0	5.6	5.3	5.1	5.5
18:2	15.3	14.8	15.0	14.8	15.1	15.5	16.4	16.1
18:1	3.3	3.1	3.2	3.2	3.4	3.8	3.9	3.7
18:2-18:2	2.1	1.8	2.2	1.9	1.7	1.4	1.9	1.8
18:1-20:4	0.7	0.8	0.9	0.9	1.1	0.8	1.0	0.7
18:2	2.3	2.3	2.4	2.4	2.1	2.1	2.2	2.2
16:0-16:1[b]								
	1.7	1.7	1.7	1.0	1.0	0.6	1.9	1.8
18:1-22:6[b]								

[a]For experimental details, see legend of Table 5. Phosphatidylcholine molecular species were determined as indicated in the Materials and Methods section. The results represent one determination. See Table 1 for other abbreviations.
[b]Molecular species were not separated.

CDCA-induced suppression of bile acid synthesis (52,53), resulting from the increase in the relative percentage of CDCA in bile, in both CDCA- and UDCA-fed hamsters. However, this explanation seems insufficient, in the case of UDCA feeding, which *in vivo*, increased LDL-receptor-dependent LDL uptake (26). A more important factor is probably the extensive washing of the cells during hepatocyte isolation which would remove UDCA from the plasma membrane (27), and thus eliminate any direct effect of this chronically fed bile acid on the LDL receptor. However, as shown in the current study, acute exposure, which would occur with each passage of UDCA through the liver, enhances hepatic LDL binding.

Previous studies from this laboratory (27), as well as the findings in the present study, indicate that the increase of cell-associated LDL by UDCA was not related to a detergent or toxic effect of this bile acid on the membrane. A two-hour incubation with 700 μM UDCA had no significant effect on either the lipid composition of the hepatocytes or the release of cellular lipids into the medium, indicating that the effect of UDCA on hepatic LDL binding is not due to an alteration in the membrane lipid composition. These results are supported by those of several studies, which showed that, up to very high concentrations, UDCA is not only nontoxic, but also shows evidence for preventing the cytotoxicity induced by other bile acids, such as CDCA and lithocholic acid (54–56).

Rudling and Angelin (23) have shown that the LDL binding activity of hepatic LDL receptors was stimulated three hours after acute glucagon administration in rats. However, the absence of upregulation of the LDL receptor activity at the transcriptional level, in spite of an increase in the LDL receptor number, prompted these authors to conclude that the effect was due to either a glucagon-induced decrease in LDL receptor recycling time, or a modification in the intracellular fate of the LDL receptor. In our previous studies, the UDCA-induced stimulation of LDL was found to occur within a time period during which a UDCA-induced effect on LDL receptor cycling could occur (27). However, in the present study, the ability of UDCA to increase the maximum LDL binding to isolated liver membranes provides evidence for an initial action of this bile acid at the plasma membrane level in the absence of an effect on LDL receptor processing or cycling.

The LDL receptor binding affinity in liver membranes was two- to fourfold lower than that observed in isolated hepatocytes. This may be due to the absence, in membrane vesicle preparations, of the structural support of cytoskeletal elements which may serve to anchor the LDL receptor in the intact cells, thus maintaining a certain binding conformation. The observation that the effect of UDCA on LDL binding to liver membranes is accompanied by a UDCA-induced decrease in LDL receptor binding affinity further supports the idea that the bile acid may be interacting with the receptor. Indeed, if UDCA were interacting with the LDL receptor in the cell membrane, as is our hypothesis, such an association should undoubtedly alter the affinity of this membrane-spanning receptor for its ligand, in an isolated membrane preparation, in which cytoskeletal anchors are absent.

It has also been suggested that the use of collagenase in the isolation of hepatocytes may alter receptor-dependent LDL binding (57). The results of the current studies suggest that, at the concentration used, collagenase treatment of isolated liver membranes did not significantly affect the LDL binding characteristics nor did it decrease the number of LDL receptors present at the membrane level.

Changes in the size and relative TG concentration of the LDL particle itself have been shown to affect its receptor-dependent catabolism (24,25). However, we have previously shown that the effect of UDCA was not due to either an acute alteration of the LDL particle or a change in nonspecific LDL

binding (27). Likewise, it was shown in the present study that neither UDCA nor CDCA feeding for four weeks significantly altered the relative protein, cholesterol, PL, and TG composition of the LDL particle. Lipid transfer activity among lipoprotein fractions has been shown to be present in hamster plasma (58); however, the relative composition of the HDL fraction was also not affected by bile acid feeding for four weeks, thus excluding any indirect effects of bile acids on LDL particle composition through lipid transfer.

With the use of electrophoretic methods to ascertain any effects on apoproteins, no apparent changes in either the electrophoretic mobility of the LDL and HDL particles on agarose gels, or their apoprotein composition on SDS–PAGE, were observed. Therefore, it can be assumed that neither the protein nor the PL content of the lipoprotein coat is affected by UDCA, since the respective relative charge of the LDL and HDL particles, as assessed by agarose gel electrophoresis, was not changed after chronic treatment with this bile acid. Furthermore, since all LDL used in this study was isolated at a density of 1.020–1.060, a difference in the size of the LDL particles was not a factor in the observed effect of UDCA on LDL binding. Similarly, since the relative protein and lipid concentrations of the LDL particles were not affected by chronic feeding of UDCA, the density of the lipoprotein particle should not be altered by this bile acid. Therefore, it can be concluded that the stimulatory effect of UDCA on hepatocellular LDL binding is not due to modification of the lipoprotein particle.

The observed effect of UDCA on LDL binding can occur in the absence of an effect on either LDL receptor transcriptional regulation, LDL receptor recycling, LDL particle composition or membrane lipid composition or release. It was previously postulated that the mechanism of action of UDCA may be to uncover, or recruit a latent pool of LDL receptors on the cell membrane (27). As proposed by Triggle (59), cryptic receptors may act as modulators of cell sensitivity to external stimuli. The speculation that UDCA probably uncovers receptors present at the membrane level, which normally may not be able to bind the LDL particle, is supported by the observations in the present studies. UDCA may either induce conformational allosteric changes leading to exposure of latent binding sites or make receptors, which are sequestered in the plasma membrane, accessible.

The mechanism of action by which UDCA may recruit cryptic LDL receptors could be related to its unique localization in the plasma membrane. Güldütuna *et al.* (54) have postulated that UDCA and its conjugates localize to specific regions in the membrane lipid bilayer, resulting in an increased membrane stabilization. In the present studies, only unconjugated UDCA was used; however, in previous studies (27) we have demonstrated that the only bile acids able to directly enhance LDL metabolism were UDCA and its taurine (T) and glycine (G) conjugates. UDCA, TUDCA, and GUDCA were all able to increase LDL binding by around 30%. However, GUDCA was significantly slower than UDCA and TUDCA in eliciting this effect. Moreover, similar effects have been observed with UDCA and TUDCA on hepatocellular calcium mobilization and phosphorylase a activation, although GUDCA did not mimic their effect (38). Therefore, while the taurine conjugate of UDCA appears to mimic the action of the free bile acid, the slower effect of GUDCA on hepatic LDL binding and uptake (even though equal in magnitude), may indeed be due to a differential localization of the bile acid in the plasma membrane bilayer. Collectively, these potential membrane stabilization mechanisms may be responsible for the observed effects of UDCA and its conjugates on hepatic LDL binding. It is easy to imagine how the integration of these bile acids into the plasma membrane lipid bilayer could affect the function of certain integral membrane proteins, such as the LDL receptor.

In conclusion, the present studies have focused on direct and novel mechanisms of LDL receptor regulation by bile acids which, in spite of their potential importance, have hitherto received little attention. The results of the present study support previous studies which showed that UDCA directly increases receptor-dependent LDL metabolism in isolated hamster hepatocytes. The effect of UDCA is not due to its effect on either the LDL particle, or the membrane lipid composition, and, as such, is not due to a detergent effect of the bile acid. Since UDCA has been shown to localize to certain regions in the plasma membrane lipid bilayer, its action on LDL binding and uptake may be to uncover a cryptic pool of LDL receptors in the plasma membrane, and make them accessible for binding.

ACKNOWLEDGMENTS

The authors are grateful to Dr. M.M. Cassidy, Department of Physiology, The George Washington University, for helpful discussions. The authors thank Ken Kim, Marie Maralit, Kathleen Caslow, and Joan Fasulo for their assistance in the studies with isolated hepatocyte preparations, and Dr. V. Hu and Dr. L. Gallo, Department of Biochemistry and Molecular Biology, The George Washington University, for allowing the use of their equipment. The studies were supported, in part, by grants from the American Heart Association Nation's Capital Affiliate (B.B.) and the General Medical Research Service of the Veterans Administration (S.J.R.). S.C. was a recipient of an American Liver Foundation Student Research Fellowship, and an American Heart Association Postdoctoral Fellowship (Nation's Capital Affiliate). Portions of this manuscript work were derived from a dissertation presented by S.C. to the Columbian College and Graduate School of Arts and Sciences of The George Washington University.

REFERENCES

1. Brown, M.S., and Goldstein, J.L. (1986) A Receptor-Mediated Pathway for Cholesterol Homeostasis, *Science 232,* 34–47.
2. Vega, G.L., and Grundy, S.M. (1987) Mechanisms of Primary Hypercholesterolemia in Humans, *Am. Heart J. 113,* 493–502.
3. Mahley, R.W., Hui, D.Y., and Innerarity, T.L. (1981) Two Independent Lipoprotein Receptors on Hepatic Membranes of Dog, Swine, and Man, *J. Clin. Invest. 68,* 1197–1206.
4. Turley, S.D., and Dietschy, J.M. (1988) The Metabolism and Excretion of Cholesterol by the Liver, in *The Liver: Biology and Pathology,* (Arias, I.M., Jakoby, W.B., Popper, H., Schachter,

D., and Shafritz, D.A., eds.) pp. 617–641, Raven Press, New York.
5. Spady, D.K., Turley, S.D., and Dietschy, J.M. (1985) Rates of Low Density Lipoprotein Uptake and Cholesterol Synthesis Are Regulated Independently in the Liver, *J. Lipid Res. 26,* 465–472.
6. Goldstein, J.L., and Brown, M.S. (1976) The LDL Pathway in Human Fibroblasts: A Receptor-Mediated Mechanism for the Regulation of Cholesterol Metabolism, *Curr. Top. Cell Regul. 11,* 147–181.
7. Goldstein, J.L., and Brown, M.S. (1977) The Low-Density Lipoprotein Pathway and Its Relation to Atherosclerosis, *Ann. Rev. Biochem. 46,* 897–930.
8. Brown, M.S., and Goldstein, J.L. (1983) Lipoprotein Receptors in the Liver, *J. Clin. Invest. 72,* 743–747.
9. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1975) Receptor-Dependent Hydrolysis of Cholesteryl Esters Contained in Plasma Low Density Lipoprotein, *Proc. Natl. Acad. Sci. U.S.A. 72,* 2925–2929.
10. Brown, M.S., Kovanen, P.T., and Goldstein, J.L. (1979) Receptor-Mediated Uptake of Lipoprotein-Cholesterol and Its Utilization for Steroid Synthesis in the Adrenal Cortex, *Recent Prog. Horm. Res. 35,* 215–257.
11. Spady, D.K., and Dietschy, J.M. (1985) Dietary Saturated Triacylglycerols Suppress Hepatic Low Density Lipoprotein Receptor Activity in the Hamster, *Proc. Natl. Acad. Sci. U.S.A. 82,* 4526–4530.
12. Davidson, N.O., Kollmer, M.E., and Glickman, R.M. (1986) Apolipoprotein B Synthesis in Rat Small Intestine: Regulation by Dietary Triglyceride and Biliary Lipid, *J. Lipid Res. 27,* 30–39.
13. Woollett, L.A., D.K. Spady, and Dietschy, J.M. (1992) Saturated and Unsaturated Fatty Acids Independently Regulate Low Density Lipoprotein Receptor Activity and Production Rate, *J. Lipid Res. 33,* 77–88.
14. Woollett, L.A., Spady, D.K., and Dietschy, J.M. (1992) Regulatory Effects of the Saturated Fatty Acids 6:0 Through 18:0 on Hepatic Low Density Lipoprotein Receptor Activity in the Hamster, *J. Clin. Invest. 89,* 1133–1141.
15. Ma, P.T.S., Gil, G., Sudhof, T.C., Bilheimer, D.W., Goldstein, J.L., and Brown, M.S. (1986) Mevinolin, an Inhibitor of Cholesterol Synthesis, Induces mRNA for Low Density Lipoprotein Receptor in Livers of Hamsters and Rabbits, *Proc. Natl. Acad. Sci. U.S.A. 83,* 8370–8373.
16. Hoeg, J.M., Maher, M.B., Zech, L.A., Bailey, K.R., Gregg, R.E., Lackner, K.J., Fojo, S.S., Anchors, M.A., Bojanovski, M., Sprecher, D.L., and Brewer Jr., H.B. (1986) Effectiveness of Mevinolin on Plasma Lipoprotein Concentrations in Type II Hyperlipoproteinemia, *Am. J. Cardiol. 57,* 933–939.
17. Kempen, H.J., Van Son, K., Cohen, L.H., Griffioen, M., Verboom, H., and Havekes, L. (1987) Effect of Ketoconazole on Cholesterol Synthesis and on HMG-CoA Reductase and LDL-Receptor Activities in HepG2 Cells, *Biochem. Pharmacol. 36,* 1245–1249.
18. Gupta, A.K., Sexton, R.C., and Rudney, H. (1990) Differential Regulation of Low Density Lipoprotein Suppression of HMG-CoA Reductase Activity in Cultured Cells by Inhibitors of Cholesterol Biosynthesis, *J. Lipid Res. 31,* 203–215.
19. Kovanen, P.T., Bilheimer, D.W., Goldstein, J.L., Jaramillo, J.J., and Brown, M.S. (1981) Regulatory Role for Hepatic Low Density Lipoprotein Receptors *in Vivo* in the Dog, *Proc. Natl. Acad. Sci. U.S.A. 78,* 1994–1998.
20. Kume, N., Kita, T., Mikami, A., Yokode, M., Ishii, K., Nagano, Y., and Kawai, C. (1989) Induction of mRNA for Low-Density Lipoprotein Receptors in Heterozygous Watanabe Heritable Hyperlipidemic Rabbits Treated with CS-514 (Pravastatin) and Cholestyramine, *Circulation 79,* 1084–1090.
21. Bihain, B.E., Deckelbaum, R.J., Yen, F.T., Gleeson, A.M., Carpenter, Y.A., and Witte, L.D. (1989) Unesterified Fatty Acids Inhibit the Binding of Low Density Lipoproteins to the Human Fibroblast Low Density Lipoprotein Receptor, *J. Biol. Chem. 264,* 17316–17321.
22. Kuo, P., Weinfeld, M., and Loscalzo, J. (1990) Effect of Membrane Fatty Acyl Composition on LDL Metabolism in Hep G2 Hepatocytes, *Biochemistry 29,* 6626–6632.
23. Rudling, M., and Angelin, B. (1993) Stimulation of Rat Hepatic Low Density Lipoprotein Receptors by Glucagon. Evidence of a Novel Regulatory Mechanism in Vivo, *J. Clin. Invest. 91,* 2796–2805.
24. Chappell, D.A., Fry, G.L., Waknitz, M.A., and Berns, J.J. (1991) Ligand Size as a Determinant for Catabolism by the Low Density Lipoprotein (LDL) Receptor Pathway. A Lattice Model for LDL Binding, *J. Biol. Chem. 266,* 19296–19302.
25. McKeone, B.J., Patsch, J.R., and Pownall, H.J. (1993) Plasma Triglycerides Determine Low Density Lipoprotein Composition, Physical Properties, and Cell-Specific Binding in Cultured Cells, *J. Clin. Invest. 91,* 1926–1933.
26. Malavolti, M., Fromm, H., Ceryak, S., and Roberts, I.M. (1987) Modulation of Low Density Lipoprotein Receptor Activity by Bile Acids: Differential Effects of Chenodeoxycholic and Ursodeoxycholic Acids in the Hamster, *J. Lipid Res. 28,* 1281–1295.
27. Bouscarel, B., Fromm, H., Ceryak, S., and Cassidy, M.M. (1991) Ursodeoxycholic Acid Increases Low-Density Lipoprotein Binding, Uptake and Degradation in Isolated Hamster Hepatocytes, *Biochem. J. 280,* 589–598.
28. Exton, J.H. (1975) The Perfused Rat Liver, *Methods Enzymol. 37,* 23–40.
29. Redgrave, T.G., Roberts, D.C.K., and West, C.E. (1975) Separation of Plasma Lipoproteins by Density-Gradient Ultracentrifugation, *Anal. Biochem. 65,* 42–49.
30. Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 98,* 231–237.
31. Irwin, D., O'Looney, P.A., Quinet, E., and Vahouny, G.V. (1984) Application of SDS Gradient Polyacrylamide Slab Gel Electrophoresis to Analysis of Apolipoprotein Mass and Radioactivity of Rat Lipoproteins, *Atherosclerosis 53,* 163–172.
32. Switzer, R.C., Merril, C.R., and Shifrin, S. (1979) A Highly Sensitive Silver Stain for Detecting Proteins and Peptides in Polyacrylamide Gels, *Anal. Biochem. 98,* 231–237.
33. Elevitch, F.R., Aronson, S.B., Feichtmeir, T.V., and Enterline, M.L. (1966) Thin Gel Electrophoresis in Agarose, *Am. J. Clin. Path. 46,* 692–697.
34. Papadopoulos, N.M., and Kintzios, J.A. (1969) Determination of Human Serum Lipoproteins by Agarose Gel Electrophoresis, *Anal. Biochem. 30,* 421–426.
35. Fromm, H., Carlson, G.L., Hofmann, A.F., Farivar, S., and Amin, P. (1980) Metabolism in Man of 7-Ketolithocholic Acid: Precursor of Cheno- and Ursodeoxycholic Acids, *Am. J. Physiol.,* G161–G166.
36. McFarlane, A.S. (1958) Efficient Trace-Labelling of Proteins with Iodine, *Nature (London) 182,* 53.
37. Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) The Metabolism of Very Low Density Lipoprotein Proteins. I. Preliminary *in Vivo* and *in Vitro* Observations, *Biochim. Biophys. Acta 260,* 212–221.
38. Bouscarel, B., Fromm, H., and Nussbaum, R. (1993) Ursodeoxycholate Mobilizes Intracellular Ca^{2+} and Activates Phosphorylase a in Isolated Hepatocytes, *Am. J. Physiol. 264,* G243–G251.
39. Prpic, V., Green, K.C., Blackmore, P.F., and Exton, J.H. (1984) Vasopressin-, Angiotensin II-, and α_1-Adrenergic-Induced Inhibition of Ca^{2+} Transport by Rat Liver Plasma Membrane Vesicles, *J. Biol. Chem. 259,* 1382–1385.

40. Kovanen, P.T., Brown, M.S., and Goldstein, J.L. (1979) Increased Binding of Low Density Lipoprotein to Liver Membranes from Rats Treated with 17α-Ethinyl Estradiol, *J. Biol. Chem. 254,* 11367–11373.
41. Scatchard, G. (1949) The Attraction of Proteins for Small Molecules and Ions, *Ann. NY Acad. Sci. 51,* 660–672.
42. McPherson, G.A. (1985) *Kinetic, EBDA, Ligand, Lowry. A Collection of Radioligand Binding Analysis Programs,* pp. 14–96, Elsevier Science Publishers BV, Amsterdam.
43. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37,* 911–917.
44. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1990) Analysis of Lipids by High-Performance Liquid Chromatography: Part I, *J. Nutr. Biochem. 1,* 493–500.
45. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1990) Analysis of Lipids by High-Performance Liquid Chromatography. Part II: Phospholipids, *J. Nutr. Biochem. 1,* 549–556.
46. Bartlett, G.R. (1959) Phosphorus Assay in Column Chromatography, *J. Biol. Chem. 234,* 466–468.
47. Singhal, A.K., Finver-Sadowsky, J., McSherry, C., and Mosbach, E.H. (1983) Effect of Cholesterol and Bile Acids on the Regulation of Cholesterol Metabolism in the Hamster, *Biochim. Biophys. Acta 752,* 214–222.
48. Fromm, H., Roat, J.W., Gonzalez, V., Sarva, R.P., and Farivar, S. (1983) Comparative Efficacy and Side Effects of Ursodeoxycholic and Chenodeoxycholic Acids in Dissolving Gallstones, *Gastroenterology 85,* 1257–1264.
49. Leiss, O., Von Bergmann, K., Streicher, U., and Strotkoetter, H. (1984) Effect of Three Different Dihydroxy Bile Acids on Intestinal Cholesterol Absorption in Normal Volunteers, *Gastroenterology 87,* 144–149.
50. Nielsell, K.B., Angelin, B., Leijd, B., and Einarsson, K. (1983) Comparative Effects of Ursodeoxycholic Acid and Chenodeoxycholic Acid on Bile Acid Kinetics and Biliary Lipid Secretion in Humans, *Gastroenterology 85,* 1248–1256.
51. Angelin, B., Raviola, C.A., Innerarity, T.L., and Mahley, R.W. (1983) Regulation of Hepatic Lipoprotein Receptors in the Dog. Rapid Regulation of Apolipoprotein B,E Receptors, But Not of Apolipoprotein E Receptors, by Intestinal Lipoproteins and Bile Acids, *J. Clin. Invest. 71,* 816–831.
52. Duckworth, P.F., Vlahcevic, Z.R., Studer, E.J., Gurley, E.C., Heuman, D.M., Beg, Z.H., and Hylemon, P.B. (1991) Effect of Hydrophobic Bile Acids on 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Activity and mRNA Levels in the Rat, *J. Biol. Chem. 266,* 9413–9418.
53. Shefer, S., Nguyen, L.B., Salen, G., Ness, G.C., Chowdhary, I.R., Lerner, S., Batta, A.K., and Tint, G.S. (1992) Differing Effects of Cholesterol and Taurocholate on Seady-State Hepatic HMG-CoA Reductase and Cholesterol 7α-Hydroxylase Activities and mRNA Levels in the Rat, *J. Lipid Res. 33,* 1193–1200.
54. Güldütuna, S., Zimmer, G., Imhof, M., Bhatti, S., You, T., and Leuschner, U. (1993) Molecular Aspects of Membrane Stabilization by Ursodeoxycholate, *Gastroenterology 104,* 1736–1744.
55. Leuschner, U., Fischer, H., Kurtz, W., Güldütuna, S., Hübner, K., Hellstern, A., Gatzen, M., and Leuschner, M. (1989) Ursodeoxycholic Acid in Primary Biliary Cirrhosis: Results of a Controlled Double Blind Trial, *Gastroenterology 97,* 1268–1274.
56. Galle, P.R., Theilmann, L., Raedsch, R., Otto, G., and Stiehl, A. (1990) Ursodeoxycholate Reduces Hepatotoxicity of Bile Salts in Primary Human Hepatocytes, *Hepatology 12,* 486–491.
57. Havel, R.J. (1986) Role of the Liver in Lipoprotein Catabolism, *Methods Enzymol. 129,* 591–612.
58. Quig, D.W., Arbeeny, C.M., and Zilversmit, D.B. (1991) Effect of Hyperlipidemias in Hamsters on Lipid Transfer Protein Activity and Unidirectional Cholesteryl Ester Transfer in Plasma, *Biochim. Biophys. Acta 1083,* 257–264.
59. Triggle, D.J. (1982) Receptor Recruitment and Cryptic Signals, *Trends Pharmacol. Sci. 3,* 273–274.

[Received October 5, 1994, and in revised form April 3, 1995; Revision accepted April 20, 1995]

Carbohydrate Type and Amount Alter Intravascular Processing and Catabolism of Plasma Lipoproteins in Guinea Pigs

Maria Luz Fernandez*, A. Karin Conde, Laura R. Ruiz, Carlos Montano, John Ebner and Donald J. McNamara

Department of Nutritional Sciences and Interdisciplinary Nutritional Sciences Program, University of Arizona, Tucson, Arizona 85721

ABSTRACT: To test the effects of exchanging dietary complex and simple carbohydrate for fat calories on lipoprotein metabolism, guinea pigs were fed two different fat/carbohydrate ratios: 2.5:58% (w/w) or 25:29% (w/w) with either sucrose or starch as the carbohydrate source. Animals fed high-fat had higher plasma low-density lipoprotein (LDL) and hepatic cholesterol concentrations than animals fed low-fat diets ($P < 0.01$). The cholesteryl ester content per particle was higher, and the number of triacylglycerol (TAG) molecules was lower in very low density lipoprotein (VLDL) and LDL from animals fed high-fat diets. Intake of high-fat/sucrose resulted in higher plasma LDL concentrations than intake of high-fat/starch, and animals fed low-fat/starch had the highest plasma TAG concentrations associated with VLDL particles containing more TAG molecules, as well as a TAG-enriched LDL. The activity of plasma lecithin cholesteryl:acyl transferase (LCAT) was highest in animals fed high-fat/sucrose, and heart lipoprotein lipase (LPL) activity was higher in animals fed high-fat diets. Hepatic apoprotein B/E (apo B/E) receptor number (B_{max}) was increased 21% with low-fat diets ($P < 0.01$). These results suggest that the hypercholesterolemia induced by high-fat and by sucrose intake are associated with a higher plasma LCAT activity which results in a cholesteryl ester-enriched VLDL which, by the action of LPL, might be more readily converted to LDL through the delipidation cascade leading to downregulation of hepatic apo B/E receptors. The hypertriglyceridemia associated with low-fat intake may result from increased production of VLDL TAG, which would explain the increased TAG content and the higher TAG/CE ratio of VLDL from animals fed the low-fat/starch diet.
Lipids 30, 619–626 (1995).

High-carbohydrate, low-fat diets have been shown to decrease plasma low-density lipoprotein (LDL) concentrations (1); however, they can also increase plasma triacylglycerol (TAG) and decrease high-density lipoprotein (HDL) concentrations (2,3), resulting in a plasma lipid profile associated with increased risk for cardiovascular disease (1). For example, diets containing 55–60% of calories derived from carbohydrates have been shown to increase plasma TAG levels in hyperlipidemic and noninsulin-dependent diabetes mellitus patients (4,5). Other studies have shown that, compared to a high-fat diet (43% calories from carbohydrates), intake of a low-fat diet (60% carbohydrate calories) results in a lowering of plasma HDL cholesterol levels independent of the type of dietary fatty acids (6,7). In these studies, an increase in plasma TAG was only observed when patients were fed simple sugars in a liquid formula (6). Studies in normolipidemic subjects have shown a progressive increase in plasma TAG levels as sucrose intake was increased (8). It has also been demonstrated that the metabolic effects of high carbohydrate diets related to increased plasma TAG concentrations can be attenuated if dietary sucrose is not increased in parallel (9).

Animal studies have been contradictory regarding the plasma lipid response to dietary carbohydrates. Although simple carbohydrates have been consistently shown to increase plasma TAG concentrations in normal and in Zucker rats (10–12), studies in cynomolgus monkeys showed no effect on plasma TAG levels with consumption of 49 vs. 76.5% of calories derived from sucrose or starch (13,14).

The effects of exchanging carbohydrate calories with fat have been studied in guinea pigs (15). Increasing dietary fat resulted in higher plasma LDL concentrations, which could be explained, in part, by increases in hepatic cholesterol concentrations and decreases in hepatic apoprotein B/E (apo B/E) (LDL) receptor number (B_{max}) (15). Studies in the guinea pig have also demonstrated that a high sucrose intake raises plasma TAG compared to starch, and while TAG secretion rates were not modified by the type of carbohydrate (16), the higher plasma TAG could be explained by increased apo B secretion rates in animals fed the simple carbohydrate.

Although interactions between the type and amount of dietary carbohydrates have been previously addressed (9), the present studies were undertaken to differentiate carbohydrate type/amount specific effects on the mechanisms which deter-

*To whom correspondence should be addressed at Department of Nutritional Sciences, Shantz Building 309, University of Arizona, Tucson, AZ 85721.

Abbreviations: Apo, apolipoprotein; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; HL, hepatic lipase; LCAT, lecithin cholesteryl:acyl transferase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; TAG, triacylglycerol; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

mine plasma lipid levels. The guinea pig was used as the animal model for this investigation, based on its plasma lipoprotein profile (high LDL/HDL ratio); distribution of hepatic cholesterol pools, activities of plasma lecithin cholesteryl:acyl transferase (LCAT) and cholesteryl ester transfer protein (CETP) and of tissue lipoprotein lipase (LPL), which are involved in the intravascular processing of lipoproteins; and the documented responses to dietary treatments, which are similar to humans (15–17).

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources—cholesterol and TAG enzymatic assay kits, cholesterol oxidase, and cholesterol esterase from Boehringer-Mannheim (Indianapolis, IN), free cholesterol and phospholipid enzymatic assay kits from Waco Pure Chemical Industries, Ltd. (Osaka, Japan), ^{121}I-Na from Amersham (Clearbrook, IL), halothane from Halocarbon (Hackensack, NJ), and quickseal ultracentrifugation tubes from Beckman Instruments (Palo Alto, CA).

Animals. Male Hartley guinea pigs (Sprague Dawley; Sasco, Omaha, NE) weighing between 300–400 g were randomly assigned to one of four experimental diets for four weeks to achieve a metabolic steady state prior to analysis of the metabolic variables. Animals were kept in a light-cycled room, fed *ad libitum* and gradually adapted to the experimental diets by mixing regular chow and increasing portions of experimental diets in a six-day period. At the end of the four-week experimental period, animals were fasted overnight, anesthetized with halothane vapors, and exsanguinated by cardiac puncture. Animals from the four dietary groups consumed equal amounts of diet, and there were no significant differences in the rates of weight gain or final weights between groups. Animal experiments were conducted in accordance with U.S. Public Health Service and U.S. Department of Agriculture guidelines, and experimental protocols were approved by the University of Arizona Institutional Animal Care and Use Committee.

TABLE 1
Composition of Experimental Diets

	Low-fat		High-fat	
	g/100 g	% Calories	g/100 g	% Calories
Protein	18.8	22.6	24.0	22.6
Fat[a]	2.5	6.9	25.1	53.0
Carbohydrate[b]	59.3	70.6	26.2	24.4
Fiber	11.0	—	14.0	—
Vitamins	1.0	—	1.2	—
Minerals	7.3	—	9.0	—
Cholesterol	0.033	—	0.038	—
Nutrient caloric density (g/1,000 kcal)				
Protein	56.1		56.2	
Fiber	32.8		32.8	
Vitamins[c]	2.99		2.81	
Minerals[c]	21.8		21.1	
Cholesterol	0.094		0.094	

[a]Fat mix: palm oil/butter fat (1:1).
[b]Carbohydrate either sucrose or corn starch.
[c]Compositions of vitamins and minerals mixtures have been previously reported (Ref. 16).

Diets. Diets were prepared and pelleted by Research Diets, Inc. (New Brunswick, NJ). Two levels of dietary fat were used—2.5 and 25% (w/w) corresponding to 6.9 and 53% of total calories with levels of carbohydrate at either 59.3 or 26.2% (w/w) equivalent to 70.6 and 24.4% of total calories (Table 1). The dietary carbohydrate was either sucrose or starch. The fat source was a mix of palm oil/butter fat (1:1), with the following fatty acid composition: myristic, 6.8%; palmitic, 33.6%; oleic, 32%; and linoleic, 7.8% (4:3:1 polyunsaturated/monounsaturated/saturated fat ratio). The amounts of fiber, protein, vitamins, and minerals were adjusted to an identical caloric density so that animals consumed similar amounts of nonfat, noncarbohydrate nutrients to assure that any observed changes were attributable to the amount and type of carbohydrate in the diet. The diets contained 0.04% cholesterol which, for the guinea pig, is the physiological equivalent (i.e., absorbed cholesterol relative to endogenous cholesterol synthesis) of 300 mg/d for humans (17).

Lipoprotein isolation. Plasma samples were obtained from guinea pigs under halothane vapor by cardiac puncture; EDTA was used as an anticoagulant (1 mg/mL), and aprotinin (0.5 mg/mL), phenylmethylsulfonylfluoride (0.1 mg/mL), and sodium azide (0.1 mg/mL) were added to minimize changes in lipoprotein composition during isolation. Plasma lipoproteins were isolated by sequential ultracentrifugation with the following densities: very low density lipoprotein (VLDL) (d = 1.006 g/mL) and LDL (d = 1.019–1.09 g/mL) as previously described (14,15). Plasma cholesterol and TAG were determined by enzymatic assays and HDL cholesterol by measuring cholesterol in the supernatant after precipitation of apo B lipoproteins with dextran sulfate–$MgCl_2$ (18).

Chemical determinations. Plasma total and lipoprotein cholesterol concentrations and plasma TAG were measured using enzymatic kits and a microtiter plate reader (15). Hepatic total and free cholesterol and TAG were determined according to Carr *et al.* (19) after extraction of hepatic lipids with chloroform/methanol (2:1).

VLDL and LDL compositions were determined by measuring lipid components (total and free cholesterol, TAG, phospholipids) and protein by a modified Lowry procedure (20). Based on LDL composition and molecular weight of components (412,000 Da for guinea pig apo B) (21), LDL diameters were calculated according to Van Heek and Zilversmit (22). Apo B in VLDL was selectively precipitated with isopropanol (23), and based on one apo B per molecule, the number of molecules of the lipid components were calculated.

LPL assay. Heart tissue was isolated from guinea pigs, and LPL activities assayed in the supernatant following homogenization of 1 g tissue in buffer (0.025 mol ammonia, 5 mmol

EDTA, 8 mg Triton X-100/mL, 0.4 mg SDS/mL, 5 IU heparin/mL, 10 μg leupeptin/mL, 1 μg pepstatin/mL, 25 IU trasylol/mL) and centrifuged at 10,000 × *g* for 20 min at 4°C. The homogenate was incubated with [^{3}H]triolein (50×10^6 dpm) for 30 min at 25°C in a water bath, and reactions were stopped by addition of 3.25 mL methanol/chloroform/heptane (1.41:1.24:1). The methanol/water upper phase was removed, and free fatty acid radioactivity determined in a scintillation counter. Results are expressed as nmol of fatty acid released per min per gram tissue at 25°C.

LCAT assay. Freshly isolated plasma samples were incubated at 0°C (control) or 37°C (experimental) for 6 h. Plasma free cholesterol concentrations were determined in control and in incubated samples by enzymatic methods using a microtiter plate reader (19). This method measures the physiological plasma LCAT activity from the decrease in the mass of endogenous free cholesterol in incubated plasma samples. LCAT activity was expressed as the molar cholesterol esterification rate (μmol decrease in unesterified cholesterol/h-mL plasma). The conditions for plasma LCAT activity have been standardized for guinea pig (15).

LDL binding assays. Pooled samples of guinea pig LDL were radioiodinated with ^{125}I by the iodine monochloride method (24) to give a specific activity between 120–300 cpm/ng. Hepatic membranes isolated as previously described (15) from animals fed the homologous diet were incubated with the radiolabeled LDL over a concentration range of 10 to 80 μg/mL in the presence or absence of 1 mg/mL of unlabeled human LDL, an effective competitor at 37°C (25). After incubation, membranes were pelleted and counted. K_d and B_{max} were determined from Woolf plots (26).

Statistical analysis. One-way analysis of variance (ANOVA) was used to determine significant differences in plasma total and lipoprotein cholesterol and TAG concentrations, LDL and VLDL compositions, hepatic free and esterified cholesterol, plasma LCAT, heart LPL and LDL binding to hepatic membranes in animals from the four dietary groups, and the Newman-Keules post-hoc was used to identify differences in means after ANOVA (INSTAT, San Diego, CA). Two-way ANOVA was used to detect differences due to the type of carbohydrate or dietary fat amount (or percentage of dietary carbohydrate). Because animals fed low- and high-fat diets had significantly different weight gains, weight gain was used as a covariate in all determined measurements. Linear regression was used to identify significant correlations between variables.

RESULTS

Carbohydrate type and fat amount effects on plasma lipoprotein levels and composition. There were no significant differences in body weight gain in animals fed the four dietary groups as indicated by one-way ANOVA; however, animals fed high-fat diets gained more weight per day than animals fed low-fat diets (Table 2). For this reason, weight gain was used as a covariate to determine whether differences in measured parameters were affected by differences in weight gain of animals fed low- and high-fat diets.

TABLE 2
Plasma Cholesterol, Triacylgycerol, and Weight Gain of Guinea Pigs Fed 2.5 or 25% Fat with Sucrose or Starch[a]

Diet	Weight gain (g/day)	Plasma lipids (mg/dL) Cholesterol	Triacylglycerol
2.5% Fat/sucrose	6.9 ± 1.4	53.8 ± 18.0[d]	108.8 ± 34.1[c]
25% Fat/sucrose	7.7 ± 1.7	118.3 ± 26.7[b]	101.8 ± 28.7[c]
2.5% Fat/starch	6.6 ± 1.2	53.3 ± 18.5[d]	182.8 ± 79.6[b]
25% Fat/starch	7.9 ± 0.6	86.3 ± 15.8[c]	99.3 ± 42.0[c]
Two-way ANOVA			
Fat amount	$P = 0.03$	$P < 0.0001$	$P = 0.039$
Carbohydrate type	N.S.	$P = 0.06$	N.S.
Interaction	N.S.	N.S.	N.S.

[a]Values represent mean ± SD for n = 6 determinations. Values in the same column with different superscripts are significantly different as determined by analysis of variance (ANOVA) and Newman–Keules post-hoc test ($P < 0.01$). N.S., not significant.

Plasma cholesterol levels were higher in animals fed high-fat diets ($P < 0.0001$), and there was a carbohydrate effect as animals fed the high-fat/sucrose diet had higher plasma cholesterol concentrations than animals fed high-fat/starch (Table 2). Plasma TAG levels were higher in animals fed low-fat compared to high-fat diets ($P < 0.05$), and the most pronounced increase in plasma TAG was observed in animals fed the high-starch diet (Table 2).

Plasma VLDL cholesterol concentrations were higher in animals fed the high-starch diet (Table 3), which correlates with the higher plasma TAG levels observed in this group (Table 2). Plasma VLDL cholesterol levels of animals fed high-fat/sucrose were not significantly different from any of the three dietary groups (Table 3). Plasma LDL cholesterol concentrations followed an identical trend with plasma total cholesterol; high-fat diets increased plasma LDL levels with

TABLE 3
Lipoprotein Cholesterol of Guinea Pigs Fed 2.5 or 25% (w/w) Fat with Sucrose or Starch[a]

Diet	Lipoprotein cholesterol (mg/dL) VLDL	LDL	HDL[b]
2.5% Fat/sucrose	5.7 ± 1.9[b]	39.2 ± 18.3[c]	9.5 ± 2.7
25% Fat/sucrose	8.5 ± 2.8[a,b]	96.8 ± 19.8[a]	10.2 ± 3.5
2.5% Fat/starch	13.2 ± 8.2[a]	31.3 ± 14.3[c]	9.5 ± 1.9
25% Fat/starch	6.6 ± 2.5[b]	67.5 ± 11.8[b]	12.1 ± 3.6
Two-way ANOVA			
Fat amount	N.S.	$P < 0.0001$	$P = 0.07$
Carbohydrate type	N.S.	$P = 0.011$	N.S.
Interaction	0.035	N.S.	N.S.

[a]Values represent mean ± SD for n = 6 determinations. Values in the same column with different superscripts are significantly different as determined by one-way ANOVA and the Newman–Keules post-hoc test ($P < 0.01$). Abbreviations as in Table 2.

[b]High-density lipoprotein (HDL) cholesterol was measured after precipitation with $MgCl_2$ and dextran sulfate as indicated in the Materials and Methods section. VLDL, very low density lipoprotein, LDL, low-density lipoprotein.

TABLE 4
LDL Triacylglycerol (TAG), Cholesteryl Ester (CE), Phospholipid (PL), and Free Cholesterol (FC) Content of LDL Particles (n = 6) of Guinea Pigs Fed 2.5 or 25% (w/w) Fat with Sucrose or Starch[a]

Diet	LDL (molecules per particle)				LDL diameter (Å)
	CE	TAG	PL	FC	
2.5% Fat/sucrose	644 ± 157[c]	518 ± 222[c]	180 ± 62	31 ± 20[c]	288 ± 52[b,c]
25% Fat/sucrose	843 ± 75[b]	342 ± 52[c]	191 ± 35	35 ± 4[c]	277 ± 31[b,c]
2.5% Fat/starch	388 ± 400[d]	734 ± 25[b]	164 ± 55	60 ± 20[b]	342 ± 87[b]
25% Fat/starch	660 ± 98[c]	341 ± 66[c]	193 ± 25	41 ± 13[c]	243 ± 16[c]
Two-way ANOVA					
Fat amount	$P = 0.0001$	$P = 0.0004$	N.S.	N.S.	$P = 0.05$
Carbohydrate type	$P = 0.0002$	$P = 0.015$	N.S.	$P = 0.01$	N.S.
Interaction	N.S.	N.S.	N.S.	N.S.	$P = 0.03$

[a]Values represent mean ± SD for n = 6 determinations. Values in the same column with different superscripts are significantly different as determined by one-way ANOVA and the Newman–Keules post-hoc test ($P < 0.01$). Abbreviations as in Tables 2 and 3.

the high-fat/sucrose diet having the most pronounced effect (Table 3). No significant differences were observed in plasma HDL cholesterol concentrations, although there was a trend for lower concentrations in animals fed low-fat diets (Table 3).

VLDL composition was significantly modified by diet and the lipid content changed accordingly (Fig. 1). Animals fed low-fat diets exhibited larger VLDL particles containing more TAG and phospholipids than VLDL from animals fed high-fat diets (Fig. 1). The type of carbohydrate also influenced VLDL composition as animals fed sucrose had a higher cholesteryl ester content per VLDL than animals fed starch diets.

Plasma LDL size and composition were also affected by carbohydrate type and amount. Based on a single apo B per LDL, the TAG, phospholipid, free cholesterol, and cholesteryl ester molecules per particle were calculated to provide information on the composition of LDL. The purity of LDL isolated from the four dietary groups was verified by SDS–PAGE (data not shown), and only apo B was present. Cholesteryl ester content was highest in animals fed the high-fat/sucrose diet and lowest in LDL from the low-fat/starch group (Table 4). High-fat and sucrose intake resulted in a higher concentration of cholesteryl ester in LDL, as indicated by two-way ANOVA (Table 4). The TAG content of LDL was lower in animals fed high-fat diets, and a carbohydrate effect was evident as TAG content was highest in animals fed low-fat/starch, resulting in an LDL particle with higher TAG content and larger diameter than is usually found for LDL (27) (Table 4). The number of phospholipid molecules was not modified by carbohydrate type or amount, whereas carbohydrate type altered the free cholesterol content of LDL with increased values for LDL from animals fed the low-fat/starch diet. The high percentage of TAG in LDL from animals fed low-fat/starch resulted in the largest LDL particle as judged from the calculated diameters (Table 4). The higher TAG mass of LDL from the low-fat/starch group and the higher cholesteryl ester content of LDL from animals in the high-fat/sucrose group in part account for the larger diameters of these particles compared to LDL from the high-fat/starch group (Table 4).

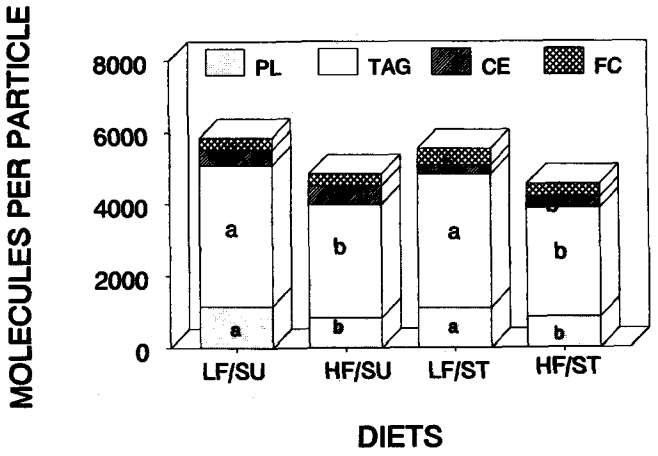


FIG. 1. Phospholipid (PL), triacylglycerol (TAG), free cholesterol (FC), and cholesteryl ester (CE) content of very low density lipoprotein particles from guinea pigs fed low-fat/sucrose (LF/SU), high-fat/sucrose (HF/SU), low-fat/starch (LF/ST), and high-fat/starch (HF/ST). The numbers of TAG and PL molecules were significantly higher in animals fed low-fat diets ($P < 0.01$), while the number of CE molecules was higher in animals fed sucrose diets ($P < 0.01$). Each bar represents the mean ± SD of n = 6 determinations.

Carbohydrate type and fat amount effects on plasma LCAT and heart LPL. Plasma LCAT activity was higher in guinea pigs fed high-fat diets, and the highest activity was found in animals fed high-fat/sucrose (Table 5). Plasma LCAT activity and VLDL cholesteryl ester content (percent) exhibited a positive correlation (Fig. 2) ($r = 0.99$, $P < 0.01$) as well as plasma LCAT and cholesteryl ester in LDL ($r = 0.97$, $P < 0.01$, data not shown), suggesting that as cholesterol is being esterified in HDL, it is rapidly transported to the apo B-containing lipoproteins, presumably by the action of CETP.

Although adipose tissue was isolated from the test animals, LPL activity could not be measured, due to the extremely low enzyme activity in fasted guinea pigs (28). LPL in the heart is

TABLE 5
Plasma Lecithin Cholesteryl:Acyl Transferase (LCAT) and Heart Lipoprotein Lipase Activity of Guinea Pigs Fed 2.5 or 25% (w/w) Fat Diets with Sucrose or Starch

Diet	Plasma LCAT (μmol/h-mL)	Heart lipoprotein lipase (nmoL/min-g)
2.5% Fat/sucrose	5.7 ± 2.0[d]	677 ± 153
25% Fat/sucrose	11.2 ± 3.1[b]	808 ± 220
2.5% Fat/starch	4.3 ± 2.3[d]	690 ± 99
25% Fat/starch	7.8 ± 0.5[c]	862 ± 153
Two-way ANOVA		
Fat amount	$P = 0.0002$	$P = 0.035$
Carbohydrate type	$P = 0.011$	N.S.
Interaction	N.S.	N.S.

[a]Values represent mean ± SD for n = 6 determinations. Values in the same column with different superscripts are significantly different as determined by one-way ANOVA and the Newman–Keules post-hoc test ($P < 0.01$). Abbreviations as in Table 2.

TABLE 6
Hepatic Cholesterol and Triacylglycerol Concentrations of Guinea Pigs Fed 2.5 or 25% Fat Diets with Sucrose or Starch[a]

Diet	Hepatic holesterol (mg/g) Free	Hepatic holesterol (mg/g) Esterified	Hepatic TAG (mg/g)
2.5% Fat/sucrose	2.9 ± 0.3[c]	0.35 ± 0.15[c]	12.6 ± 5.7
25% Fat/sucrose	3.6 ± 0.5[b]	0.34 ± 0.12[c]	23.0 ± 7.4
2.5% Fat/starch	3.0 ± 0.1[c]	0.41 ± 0.15[b]	24.9 ± 7.4
25% Fat/starch	3.1 ± 0.1[c]	0.53 ± 0.09[b]	23.6 ± 14.6
Two-way ANOVA			
Fat amount	$P = 0.006$	N.S.	N.S.
Carbohydrate type	N.S.	$P = 0.029$	N.S.
Interaction	$P = 0.043$	N.S.	N.S

[a]Values represent mean ± SD for n = 6 determinations. Values in the same column with different superscripts are significantly different as determined by one-way ANOVA and the Newman–Keules post-hoc test ($P < 0.01$). Abbreviations as in Tables 2 and 3.

not decreased by fasting and was found to be elevated in animals fed high-fat diets as compared to the low-fat groups ($P < 0.05$), which could, in part, explain the presence of smaller, TAG reduced plasma VLDL in animals fed the high-fat diets (Table 5).

Dietary carbohydrate type and fat amount effects on hepatic lipids and hepatic LDL receptor. Hepatic free cholesterol concentrations were significantly altered by the amount of dietary fat. Animals fed high-fat diets, especially with sucrose, had higher levels of hepatic free cholesterol than animals fed low-fat diets (Table 6). Hepatic esterified cholesterol levels were also significantly affected by the type of carbohydrate. Animals fed starch had higher concentrations of esterified cholesterol in the liver than animals fed sucrose diets ($P < 0.05$). Hepatic TAG concentrations were not affected by dietary carbohydrate type or amount (Table 6).

TABLE 7
Hepatic LDL Receptor Number (Bmax) and Affinity (Kd) of Guinea Pigs Fed 2.5 or 25% (w/w) Fat with Sucrose or Starch[a]

Diet	Binding parameters B_{max} (μg/mg)	Binding parameters K_d (μg/mL)
2.5% Fat/sucrose	2.32 ± 0.32[b]	34 ± 10
25% Fat/sucrose	1.89 ± 0.11[c]	26 ± 9
2.5% Fat/starch	2.15 ± 0.05[b]	27 ± 17
25% Fat/starch	1.81 ± 0.10[c]	22 ± 7
Two-way ANOVA		
Fat amount	$P = 0.001$	N.S.
Carbohydrate type	N.S.	N.S.
Interaction	N.S.	N.S.

[a]Values represent mean ± SD for n = 6 determinations. Values in the same column with different superscripts are significantly different as determined by one-way ANOVA and the Newman–Keules post-hoc test ($P < 0.01$). Abbreviations as in Table 2 and 3.

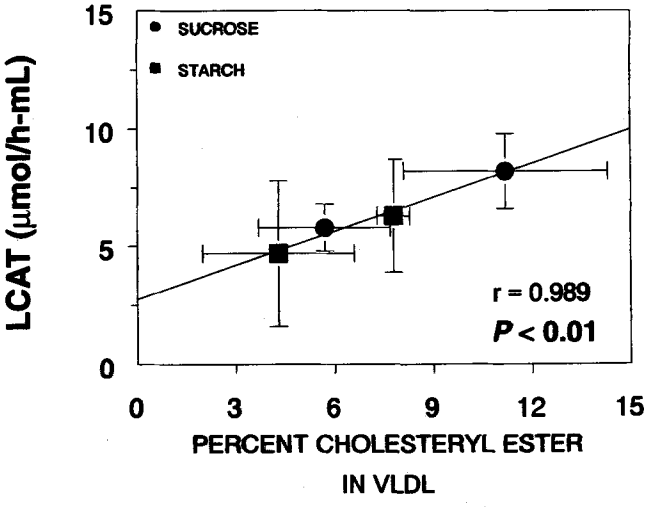


FIG. 2. Correlation between plasma lecithin cholesteryl:acyl transferase (LCAT) activity (μmol/h-mL) and percent cholesteryl ester in very low density lipoprotein (VLDL) from animals fed low-fat/starch, low-fat/sucrose, high-fat/starch, and high-fat/sucrose diets. Each point represents the mean ± SD of n = 6 determinations.

Hepatic LDL receptor B_{max} values were higher in animals fed low-fat diets, and no differences due to carbohydrate type were found (Table 7). LDL receptor affinity (K_d) was not modified by the amount of fat or the carbohydrate type (Table 7).

DISCUSSION

These studies demonstrate that, in addition to modifications in hepatic LDL receptor number, the amount of fat and type of carbohydrate in the diet affect several parameters of plasma lipoprotein production, remodeling, and catabolism which influence the concentration of plasma lipids, the composition of lipoproteins, and their potential atherogenicity.

While high-fat diets increased the plasma concentration of atherogenic LDL, intake of the low-fat diets resulted in higher concentrations of plasma TAG, a profile also associated with increased risk for cardiovascular disease. In addition, interactive effects on certain parameters of lipoprotein metabolism were observed between the type of dietary carbohydrates and the amount of dietary fat. The combination of sucrose with

high-fat intake elicited the highest plasma cholesterol levels, whereas low-fat and starch intake resulted in the highest plasma TAG concentrations. The hypertriglyceridemic effect of starch was unexpected and differs from some studies in humans (6) and in rats (29); however, other studies have found no differences in plasma TAG with intake of high-starch vs. high-sucrose diets (13,14,30). In our previous study in guinea pigs, we observed higher concentrations of plasma TAG with intake of 59% of calories from sucrose compared to starch (16). In the present study, starch contributed 70.6% of the caloric intake and was given with a highly saturated fat which, in combination, could alter the activities of various enzymes involved in lipoprotein processing, resulting in the observed pattern of responses.

Effects of dietary carbohydrates with high-fat diets. Dietary treatments significantly affected the composition of VLDL and LDL and the activity of plasma LCAT, modifications related to plasma lipoprotein remodeling in the intravascular compartment and possibly associated with plasma lipid levels and atherogenic potential. Similar to our observations, studies in rats and monkeys (10,13) have shown increases in plasma cholesterol with intake of sucrose compared to feeding a starch-based diet. Because intake of sucrose and starch at the same level of dietary fat had similar effects on hepatic LDL receptor B_{max} values, it is postulated that the interactive effects of high-fat diets with sucrose on plasma lipoprotein remodeling accounts for the higher plasma LDL levels observed in this group of animals. Studies in cynomolgus monkeys have shown that sucrose, when compared to starch intake, increases plasma LCAT and CETP and hepatic lipase (HL) activities, and that these increases are related to effects on TAG metabolism and lipoprotein interconversion processes (31). In the present study, we observed higher LCAT activity and the higher concentration of cholesteryl ester in VLDL and LDL, which suggest increased CETP activity in animals fed high-fat/sucrose; however, this would need to be measured directly to confirm this hypothesis.

Cholesteryl ester-enriched VLDL have been shown to be more readily converted to intermediate-density lipoprotein with increased conversion to LDL through the delipidation cascade (32). In addition, studies in African green monkeys have shown that cholesterol-enriched VLDL (induced by feeding a high-cholesterol diet) are readily converted to LDL, contributing to the higher plasma LDL concentrations and atherosclerosis observed in these animals (33). Based on these studies, one can speculate that these cholesteryl ester-enriched VLDL generated by high-fat/sucrose intake are more readily converted to LDL and partly account for the higher plasma LDL levels in sucrose-fed vs. starch-fed guinea pigs, given that the number of hepatic LDL receptors was not altered by the type of dietary carbohydrate. In addition, nascent VLDL from cholesterol-fed guinea pigs has been shown to have a higher concentration of cholesteryl ester than nascent VLDL from animals fed low-cholesterol diets, which appears related to the higher hepatic cholesterol concentrations (34). Because animals fed the high-fat/sucrose diet had a higher concentration of hepatic cholesterol, the increased number of cholesteryl ester molecules per VLDL from sucrose-fed animals could be explained by the increased availability of hepatic cholesterol for incorporation into nascent VLDL (34).

Effects of dietary carbohydrate with low-fat diets. With intake of both high-carbohydrate diets, significant increases in plasma TAG were observed, although the effect was more pronounced in animals fed the high-starch diets. In addition, the starch-fed guinea pigs had a large LDL with unusual compositional characteristics, indicating that the intravascular conversion of VLDL to LDL is affected by the type of dietary carbohydrate. Guinea pigs are known to have high tissue LPL activity (35), which is one reason this animal model does not develop hypertriglyceridemia under most dietary conditions; however, HL activity is low. In the present study, both low-fat groups (starch and sucrose) had similar VLDL compositions, a large particle containing a high number of TAG molecules and more phospholipids, as has been observed in rats fed high-carbohydrate diets (36). The only difference in VLDL composition between sucrose and starch diets was a higher cholesteryl ester content for the low-fat/sucrose fed guinea pigs. However, LDL composition was substantially altered by the type of carbohydrate in animals fed the low-fat diets. LPL is the key enzyme in the hydrolysis of TAG derived from VLDL and chylomicrons, whereas HL hydrolyzes phospholipids of remnant particles (VLDL remnant) and HDL (37). Guinea pigs fed starch diets had higher concentrations of hepatic cholesteryl ester than did animals fed sucrose diets. It has been demonstrated in the Zucker rat that mRNA levels of HL are suppressed by high levels of cholesteryl ester in the liver (38) with a strong association between low HL activity and high concentrations of hepatic cholesteryl ester (38). It is possible that guinea pigs fed the starch diet had a greater suppression of HL than animals fed high sucrose due to the expanded pools of hepatic cholesteryl ester, and this, in combination with the low activity of HL in guinea pigs, resulted in a TAG-enriched LDL. The relevance of this particle to atherosclerosis deserves further attention as it is not known whether these changes in composition would alter the permeability of this LDL particle across the arterial wall or make it more susceptible to oxidation. Studies by Rudel *et al.* (39) have correlated cholesteryl ester content and LDL size with coronary artery atherosclerosis in African green monkeys and found that larger cholesteryl ester-enriched LDLs derived from animals fed saturated fat are more atherogenic than smaller LDL from monkeys fed polyunsaturated fats. Similarly, LDL from pigs with genetic hypercholesterolemia have a higher concentration of cholesteryl ester, and lower TAG content than LDL derived from normal pigs (40). Because in the present study LDL from animals fed low-fat/starch contained a high proportion of TAG and less cholesteryl ester than LDL from the other dietary groups, it is not possible at this point to compare this large TAG-enriched particle with the atherogenic large cholesteryl ester-enriched LDL observed in monkeys and in mutant pigs (39,40).

From these studies we conclude that the amount of dietary fat and the type of dietary carbohydrate elicit divergent responses in terms of plasma lipid and lipoprotein profiles and the potential atherogenicity of the evolved lipoprotein patterns deserves further consideration. Suppression of hepatic LDL receptors apparently makes a major contribution to the elevated plasma LDL observed with high-fat intake, whereas the higher plasma TAG concentrations and the larger VLDL particles resulting from high-carbohydrate intake could be due to an increase in TAG secretion from the liver (41) or a decrease in TAG removal from plasma (42). The interactive effects of the type of carbohydrate with the amount of fat also resulted in specific alterations in hepatic cholesterol distribution and the intravascular processing of lipoproteins. For example, the cholesteryl ester-enriched VLDL obtained from animals fed high-fat/sucrose is probably readily converted to LDL, thus increasing plasma LDL concentrations, and animals fed low-fat/starch exhibited a large TAG enriched LDL, whose metabolic significance and relationship to atherosclerosis is not known at present. The results from these studies also indicate that not only are fat calories relevant in terms of the plasma lipid profile and potential atherogenicity of lipoproteins, but the type of carbohydrate replacing the fat is also a major determinant of the potential cardiovascular disease risk. What is also evident is that with a high-fat diet, the type of carbohydrate contributes to the degree of fat-induced hyperlipidemia.

ACKNOWLEDGMENTS

This research was supported by a Grant-in-Aid (AZ-94-GF-42) from the American Heart Association, Arizona Affiliate. We thank Henrik Steinberg for measurements of tissue lipoprotein lipase activity.

REFERENCES

1. McNamara, D.J. (1992) Dietary Fatty Acids, Lipoproteins and Cardiovascular Disease, *Adv. Food Nutr. Res. 36*, 253–351.
2. Garg, A., Grundy, S.M., and Unger, R.H. (1992) Comparison of Effects of High and Low Carbohydrate Diets on Plasma Lipoproteins and Insulin Sensitivity in Patients with Mild NIDDM, *Diabetes 41*, 1278–1285.
3. Brinton, E.A., Eisenberg, S., and Breslow, J.L. (1990) A Low-Fat Diet Decreases High Density Lipoprotein (HDL) Cholesterol Levels by Decreasing HDL Apolipoprotein Transport Rates, *J. Clin. Invest. 85*, 144–151.
4. Liu, G.C., Coulston, A.M., and Reaven, G.M. (1983) Effect of High-Carbohydrate-Low-Fat Diets on Plasma Glucose, Insulin and Lipid Responses in Hypertriglyceridemic Humans, *Metabolism 32*, 750–753.
5. Garg, A., Bantle, J.P., Henry, R.R., Coulsaton, A.M., Griver, K.A., Raatz, S.K., Brinkley, L., Chen, Y-D. I., Grundy, S.M., Huet, B.A., and Reaven, G.M. (1994) Effects of Varying Carbohydrate Content of Diet in Patients with Non-Insulin-Dependent Diabetes Mellitus, *JAMA 271*, 1421–1428.
6. Grundy, S.M., Florentin, L., Nix, D., and Whelan, M.F. (1987) Comparison of Monounsaturated Fatty Acids and Carbohydrates for Reducing Raised Levels of Plasma Cholesterol in Man, *Am. J. Clin. Nutr. 47*, 965–969.
7. Grundy, S.M. (1986) Comparison of Monounsaturated Fatty Acids and Carbohydrates for Lowering Plasma Cholesterol, *N. Engl. J. Med. 314*, 745–748.
8. Albrink, M.J., and Ulrich, I.H. (1986) Interaction of Dietary Sucrose and Fiber on Serum Lipids in Healthy Young Men Fed High Carbohydrate Diets, *Am. J. Clin. Nutr. 43*, 419–428.
9. Liu, G., Coulston, A., Hollenbeck, C., and Reaven G. (1984) The Effect of Sucrose Content in Low and High Carbohydrate Diets on Plasma Glucose, Insulin and Lipid Responses in Hypertriglyceridemic Humans, *J. Clin. Endocrinol. 59*, 636–642.
10. Bernadier, C.D., Tobin, R.B., and DeVore, V. (1979) Effects of Age, Strain, and Dietary Carbohydrate on the Hepatic Metabolism of Male Rats, *J. Nutr. 109*, 261–271.
11. Gutman, R.A., Basilico, M.Z., Bernal, C.A., Chicco, A., and Lombardo, Y.B. (1987) Long-Term Hypertriglyceridemia and Glucose Intolerance in Rats Fed Chronically an Isocaloric Sucrose-Rich Diet., *Metabolism 36*, 1013–1020.
12. Barnard, R.J., Faria, D.J., Menges, J.E., and Martin, D.A. (1993) Effects of a High-Fat, Sucrose Diet on Serum Insulin and Related Atherosclerotic Risk Factors in Rats, *Atherosclerosis 100*, 229–236.
13. Srinivasan, S.R., Radhakrishnamurthy, B., Foster, T.A., and Berenson, G.S. (1983) Divergent Responses of Serum Lipoproteins to Changes in Dietary Carbohydrate and Cholesterol in Cynomolgus Monkeys, *Metabolism* 32, 777–786.
14. Srinivasan, S.R., Radhakrishnamurthy, B., Foster, T.A., and Berenson, G.S. (1984) Serum Lipoprotein Lipid and Apoprotein (B and A-I) Responses to Saturated Fat-Rich Diet in Cynomolgus Monkeys: Effects of Carbohydrate Type and Cholesterol, *Am. J. Clin. Nutr. 40*, 485–495.
15. Fernandez, M.L., Sun, D-M., Montano, C., and McNamara, D.J. (1995) Carbohydrate-Fat Exchange and Regulation of Hepatic Cholesterol Homeostasis and Lipoprotein Metabolism, *Metabolism*, in press.
16. Fernandez, M.L., Abdel-Fattah, G., and McNamara, D.J. (1995) Differential Effects of Simple and Complex Carbohydrates on VLDL Secretion Rates and HDL Metabolism in the Guinea Pig, *Biochim. Biophys. Acta 1256*:31–38.
17. Lin, E.C.K., Fernandez, M.L., and McNamara, D.J. (1992) Dietary Cholesterol and Fat Quantity Interact to Affect Cholesterol Metabolism in Guinea Pigs, *J. Nutr. 120*, 1037–1045.
18. Warnick, G.R., Benderson, J., and Albers, J.J. (1982) Dextran-sulphate-Mg^{+2} Precipitation Procedure for Quantitation of High-Density Lipoprotein Cholesterol, *Clin. Chem. 28*, 1379–1388.
19. Carr, T., Andressen, C.J,. and Rudel, L.L. (1993) Enzymatic Determination of Triglyceride, Free Cholesterol and Total Cholesterol in Tissue Lipid Extracts, *Clin. Biochem. 26*, 39–42.
20. Markwell, M.A.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978) A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples, *Anal. Biochem. 87*, 206–210.
21. Chapman, J.M., Mills, G.L., and Ledford, J.H. (1975) The Distribution and Partial Characterization of the Serum Apolipoproteins in the Guinea Pig, *Biochem. J. 149*, 423–436.
22. Van Heek, M., and Zilversmit, D.B. (1991), Mechanisms of Hypertriglyceridemia in the Coconut Oil/Cholesterol-Fed Rabbit, *Arterioscler. Thromb. 11*, 918–927.
23. Holmsquit, L., Carlson, K., and Carlson, L.A. (1987) Comparison Between the Use of Isopropanol and Tetramethyl Urea for the Solubilization and Quantitation of Human Serum Very Low Density Apolipoproteins, *Anal. Biochem. 88*, 457–460.
24. Goldstein, J.L., Basu, S.K., and Brown, M.S. (1983) Receptor-Mediated Endocytosis of Low Density Lipoprotein in Cultured Cells, *Meth. Enzymol 98*, 241–260.
25. Fernandez, M.L., Trejo, A., and McNamara, D.J. (1990) Pectin Isolated from Prickly Pear (*Opuntia* sp.) Modifies Low Density Lipoprotein (LDL) in Cholesterol-Fed Guinea Pigs, *J. Nutr. 120*, 1283–1290.

26. Keightley, D.D., Fischer, R.J., and Cressie, N.A.C. (1983) Properties and Interpretation of the Woolf and Scatchard Plots in Analyzing Data from Steroid Receptor Assays, *J. Steroid Biochem. 9*, 1407–1412.
27. Fernandez, M.L., Abdel-Fattah, G., and McNamara, D.J. (1993) Dietary Fat Saturation Modifies the Metabolism of LDL Subfractions in Guinea Pigs, *Arterioscler. Thromb. 13*, 1418–1428.
28. Semb, H., and Olivecrona, T. (1986) Nutritional Regulation of Lipoprotein Lipase in Guinea Pig Tissues, *Biochim. Biophys. Acta 876*, 249–255.
29. Deshaies, Y., Martineau, M-J., and Lalonde, J. (1991) Chronic α_1-Adrenergic Blockade, Serum Triacylglycerols, and Tissue Lipoprotein Lipase Activity in Rats Fed Diets High in Starch or Sucrose, *Nutrition 7*, 109–115.
30. Hallfrish, J., Cohen, L., and Reiser, S. (1981) Effects of Feeding Rats Sucrose in a High Fat Diet, *J. Nutr. 111*, 531–536.
31. Sharma, C., Srinivasan, S.R., Radhakrishnamurthy, B., and Berenson, G.S. (1986) Effect of Dietary Carbohydrate Type on Lipoprotein Lipase, Hepatic Lipase, and Lecithin: Cholesterol Acyltransferase Activities in Cynomolgus Monkeys, *Biochem. Med. 36*, 51–59.
32. Nestel, P., Billington, T., Tada, N., Nugent, P., and Fidge, N. (1983) Heterogeneity of Very-Low-Density Lipoprotein Metabolism in Hyperlipidemic Subjects, *Metabolism 32*, 810–817.
33. Marzetta, C.A., Johnson, F.L., Zech, L.A., Foster, D.M., and Rudel, L.L. (1989) Metabolic Behavior of Hepatic VLDL and Plasma LDL apo B-100 in African Green Monkeys, *J. Lipid Res. 31*, 1149–1159.
34. Guo, L.S.S., Hamilton, R.L., Ostwald, R., and Havel, R.J. (1982) Secretion of Nascent Lipoproteins and Apolipoproteins by Perfused Livers of Normal and Cholesterol Fed Guinea Pigs, *J. Lipid Res. 23*, 543–555.
35. Olivecrona, T., and Bengstsson-Olivecrona, G. (1993) Lipoprotein Lipase and Hepatic Lipase, *Current Opinion Lipidology 4*, 187–196.
36. Verschoor, L., Chen, Y.D-I., Reaven, E.P., and Reaven, G.M. (1985) Glucose and Fructose Feeding Lead to Alterations in Structure and Function of Very Low Density Lipoproteins *Horm. Metabol. Res. 17*, 285–288.
37. Fielding, C.J., and Fielding, P.E. (1981) Regulation of Human Plasma Lecithin:Cholesterol Acyltransferase Activity by Lipoprotein Acceptor Cholesteryl Ester Content, *J. Biol. Chem. 256*, 2102–2110.
38. Benhizia, F., Lagrange, D., Malewiak, M-I., and Griglio, S. (1992) *In vivo* Regulation of Hepatic Lipase Activity and mRNA Levels by Diets Which Modify Cholesterol Influx to the Liver, *Biochim. Biophys. Acta 1211*, 181–188.
39. Rudel, L.L., Bond, M.G., and Bullock, B.C. (1985) LDL Heterogeneity and Atherosclerosis in Nonhuman Primates, *Ann. NY Acad. Sci. 454*, 248–253.
40. Checovich, W.J., Fitch, W.L., Krauss, R.M., Smith, M.P., Rapacz, J., Smith, C.L., and Attie, A.D. (1988) Defective Catabolism and Abnormal Composition of Low-Density Lipoproteins from Mutant Pigs with Hypercholesterolemia, *Biochem. 27*, 1934–1941.
41. Melish, J., Le, N-A., Ginsberg, H., Steinberg, D., and Brown, W.V. (1980) Dissociation of Apoprotein B and Triglyceride Production in Very Low Density Lipoproteins, *Am. J. Physiol. 239*, E354–E362.
42. Mamo, J.C.L., Hirano, T., James, L., and Steiner, G. (1991) Partial Characterization of the Fructose-Induced Defect in Very-Low-Density Lipoprotein Triglyceride Metabolism, *Metabolism 40*, 888–893.

[Received January 13, 1995, and in revised form March 21, 1995; Revision accepted April 10, 1995]

Effect of Peroxyl Radicals on Lecithin/Cholesterol Acyltransferase Activity in Human Plasma

Chen Chen and George Loo*

Department of Food, Nutrition and Food Service Management, School of Human Environmental Sciences, The University of North Carolina at Greensboro,Greensboro, North Carolina 27412-5001

ABSTRACT: The objective of this study was to determine whether subjecting human plasma to oxidant stress reduces the activity of lecithin/cholesterol acyltransferase (LCAT, EC 2.3.1.43). Plasma was incubated for 4 h with 2.25–45 mM of 2,2′-azo*bis*(2-amidinopropane)HCl (AAPH), a source of peroxyl radicals. A time- and concentration-dependent reduction of LCAT activity occurred, relative to control samples incubated in the absence of AAPH. Reduction of LCAT activity was disproportionate to elevation of thiobarbituric acid-reactive substances (TBARS) in the plasma. Added ascorbate was able to significantly prevent reduction of LCAT activity, but this effect was unrelated to blockage of TBARS formation by the antioxidant. The results suggest that LCAT activity can be down-modulated by oxidant stress, but not necessarily by lipid peroxidation.
Lipids 30, 627–631 (1995).

By modifying lipoproteins, oxidant stress may be implicated in the development of atherosclerosis (1). As low-density lipoprotein (LDL) is oxidatively modified, constituent polyunsaturated fatty acids undergo lipid peroxidation. Oxidized LDL particles are metabolized by macrophages, resulting in accumulation of cellular cholesterol. Transformation of macrophages into foam cells is an early event in the onset of atherogenesis. The impact of oxidative modification of high-density lipoprotein (HDL) is less clear. Since the bulk of lipid hydroperoxides in human plasma is believed to be carried by HDL (2), oxidation of HDL might be relevant to atherogenesis. The function of HDL may be impeded after oxidative modification. For example, oxidized HDL has a reduced capacity to promote cholesterol efflux from macrophage/foam cells (3,4).

On the other hand, HDL oxidation may adversely affect activity of the enzyme, lecithin/cholesterol acyltransferase (LCAT, EC 2.3.1.43). Plasma LCAT is typically associated with HDL and is believed to play a pivotal role in facilitating HDL-mediated removal of cholesterol from peripheral tissue sites (5). Isolated HDL activates purified LCAT, but this ability is decreased after copper-induced oxidation of HDL (6). Few studies are available concerning the effect of oxidant stress on LCAT and cholesterol esterification. The purpose of the present study was to determine if the activity of LCAT is reduced in human blood plasma exposed to a high concentration of peroxyl radicals. We also determined whether the antioxidant, ascorbate, is able to prevent the effects of peroxyl radicals on LCAT activity.

*To whom correspondence should be addressed at Department of Food, Nutrition and Food Service Management, 318 Stone Building, The University of North Carolina at Greensboro, Greensboro, NC 27412-5001.

Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane)HCl; HDL, high-density lipoprotein; LCAT, lecithin/cholesterol acyltransferase; LDL, low-density lipoprotein; TBARS, thiobarbituric acid-reactive substances.

EXPERIMENTAL PROCEDURES

Chemicals. [4-^{14}C]Cholesterol (51.40 mCi or 1.9 GBq/mmol) was purchased from New England Nuclear (Boston, MA). The hydrophilic prooxidant, 2,2′-azo*bis*(2-amidinopropane)HCl (AAPH), was obtained from Polysciences, Inc. (Warrington, PA). All other reagents were obtained from either Fisher Scientific Co. (Norcross, GA) or Sigma Chemical Co. (St. Louis, MO).

Human blood plasma. Blood was obtained by venipuncture from normal human adults using heparinized Vacutainer tubes. After centrifugation of the blood at 1,500 × *g* for 30 min at 4°C, plasma was recovered and pooled for use in the experiments.

Treatment of human blood plasma with AAPH and assay of LCAT. As detailed below, AAPH dissolved in 0.2 M sodium phosphate buffer, pH 7.4, was added to plasma while its LCAT activity was being simultaneously assayed. LCAT activity was measured using a slight modification of the method adapted by Wallentin and Vikrot (7) from the original procedure of Stokke and Norum (8). Twenty μL of 10 mM Ellman's reagent were added to test tubes containing 50 μL of plasma and 20–40 μL of 10 mM phosphate-buffered saline. The tubes were incubated for 30 min in a shaking water bath at 37°C to allow for temporary inactivation of LCAT. Then, 30 μL of [4-^{14}C]cholesterol/albumin emulsion (265,000 dpm) were added to the tubes, followed by AAPH or both AAPH and ascorbate. After 4 h of additional incubation to allow for equilibration of radiolabel, the reaction catalyzed by LCAT was initiated by addition of 20 μL of 0.1 M β-mercaptoethanol to the tubes. After 30 min, the reaction was terminated and lipids were extracted by addition of 3.4 mL of chloroform/methanol (2:1, vol/vol). Cholesteryl esters were sepa-

Copyright © 1995 by AOCS Press

rated from unesterified cholesterol by silica gel thin-layer chromatography using chloroform/methanol/glacial acetic acid/water (25:15:4:2, by vol) as the solvent system. After chromatography, iodine vapor was used to detect lipids, and appropriate spots were marked. The iodine was then allowed to completely evaporate, and spots were scraped off directly into liquid scintillation vials. After addition of 10 mL of ScintiVerse I (Fisher), radioactivity was measured in a Beckman LS 6000SE scintillation spectrometer (Fullerton, CA). In all of our work, LCAT measurements typically produce intraexperiment coefficient of variation of less than 5% and interexperiment coefficient of variation of less than 10%.

Determination of lipid peroxidation after treatment of plasma with AAPH. AAPH or both AAPH and ascorbate were added to 50–100 μL of plasma to give a total volume of 150–300 μL, depending on the experiment. Tubes were incubated at 37°C for 4.5 h. Levels of thiobarbituric acid-reactive substances (TBARS) were measured spectrophotometrically (9). Post-incubation tubes and also tubes containing 1,1,3,3-tetramethoxypropane standards (0–4.0 nmoles) received 1 mL of 20% trichloroacetic acid containing 2.15 mM EDTA. Then, 1 mL of 1% thiobarbituric acid was added. After mixing and allowing color development for 20 min in a boiling water bath, tubes were allowed to cool and were then centrifuged at 1,500 × *g* for 15 min. Absorbance of the supernatant was measured at 532 nm.

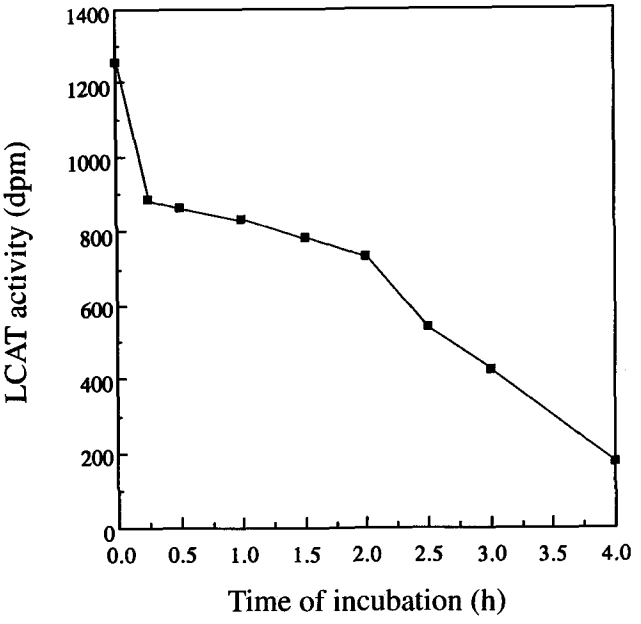


FIG. 1. Time course for the effect of 2,2′-azo*bis*(2-amidinopropane)HCl (AAPH) on the activity of lecithin/cholesterol acyltransferase (LCAT) in human blood plasma. Conditions were as in Figure 2 except that during equilibration of radiolabel, AAPH was added at various time intervals to give a final concentration of 33 mM. Counterpart control tubes received buffer alone at the beginning of the 4-h equilibration period. LCAT activity is expressed as amount of radioactivity detected in the cholesteryl ester fraction, and values are the average of two determinations.

RESULTS

Figure 1 shows the change of LCAT activity while incubating plasma with 33 mM AAPH for 4 h. With just 15 min of incubation with AAPH, there was a striking 30% reduction of LCAT activity in the plasma. Afterwards, the rate at which LCAT activity was lost remained relatively constant for up to 2 h. During this time interval of 1.75 h, only an additional 12% reduction of LCAT activity occurred. Thereafter, the rate at which LCAT activity was lost increased noticeably. At the end of 4 h after initiating the incubation, LCAT activity was reduced to 14% of control plasma samples.

As shown in Figure 2, incubation of human blood plasma with AAPH at 37°C for 4 h prior to initiating the enzyme-catalyzed reaction resulted in a concentration-dependent reduction of LCAT activity. The inhibitory effect of AAPH was small in the lower concentration range of 2.25–9 mM with only 3–13% reduction of LCAT activity. However, our preliminary data indicate that incubation of plasma at these lower AAPH concentrations for a longer period of time, instead of 4 h, produces a more appreciable reduction of LCAT activity. At the highest concentration of AAPH tested (45 mM), there was a 61% decrease in LCAT activity relative to control.

Reduction of LCAT activity in plasma incubated with AAPH was also accompanied by an increase in the levels of TBARS (Fig. 2), indicating that AAPH induced lipid peroxidation in the plasma. However, reduction of LCAT activity was only partially related to extent of lipid peroxidation. The only concentrations of AAPH that were able to produce linear increases in TBARS levels were 2.25–18 mM. These lower concentrations of AAPH also produced proportional reductions of LCAT activity. In contrast, no additional increases in TBARS levels were produced up to an AAPH concentration of 45 mM. Yet, these higher concentrations of AAPH still produced additional reduction of LCAT activity. Thus, between 18–45 mM AAPH, reduction of LCAT activity was unrelated to the extent of lipid peroxidation.

Since reduction of LCAT activity in plasma incubated with AAPH seemed to be related to lipid peroxidation at least over a certain time/concentration range, experiments were performed to evaluate the efficacy of added ascorbate to prevent both lipid peroxidation and reduction of LCAT activity. It should be noted that ascorbate concentrations that are indicated exclude the ascorbate found endogenously in normal human plasma which was not measured. However, Zannoni *et al.* (10) have reported a mean adult plasma ascorbate level of 0.071 mM, with a range of 0.012–0.121 mM. As shown in Figure 3, the presence of added ascorbate at relatively high concentrations during incubation of plasma with 30 mM AAPH was able to inhibit formation of TBARS in a concentration-dependent manner. Ascorbate added at a concentration of 8 mM was able to essentially block AAPH-induced TBARS formation under these conditions, whereas 0.5 and 1 mM ascorbate produced 21 and 46% reductions in TBARS formation, respectively. However, this concentration range of added ascorbate is much higher than would be found under physiological conditions. Therefore, the capacity of added

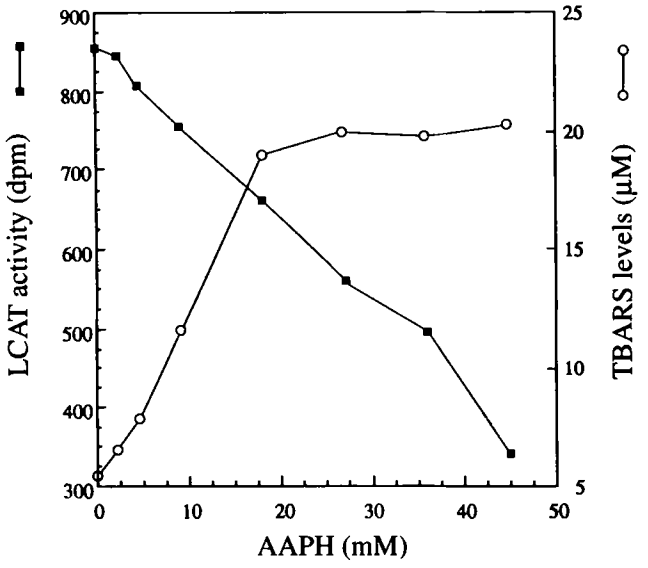


FIG. 2. Effect of 2,2'-azo*bis*(2-amidinopropane)HCl (AAPH) on the activity of lecithin/cholesterol acyltransferase (LCAT) and levels of thiobarbituric acid-reactive substances (TBARS) in human blood plasma. To test tubes containing 50 μL of plasma and 40 μL phosphate-buffered saline were added 20 μL of 10 mM Ellman's reagent. Tubes were incubated for 30 min at 37°C. Then, 30 μL of [4-^{14}C]cholesterol/albumin emulsion were added to the tubes, followed by 10 μL of AAPH to give the designated concentrations of AAPH. After 4 h of additional incubation, the reaction catalyzed by LCAT was initiated by addition of 20 μL of 0.1 M β-mercaptoethanol. LCAT activity is expressed as amount of radioactivity detected in the cholesteryl ester fraction, and values are the average of two determinations. TBARS assay was performed on other samples. To test tubes containing 100 μL of plasma and 160 μL of phosphate-buffered saline were added AAPH to give a total volume of 300 μL. Tubes were incubated at 37°C for 4.5 h, and levels of TBARS were determined as described in the Experimental Procedures section. Values represent the average of quadruplicate determinations.

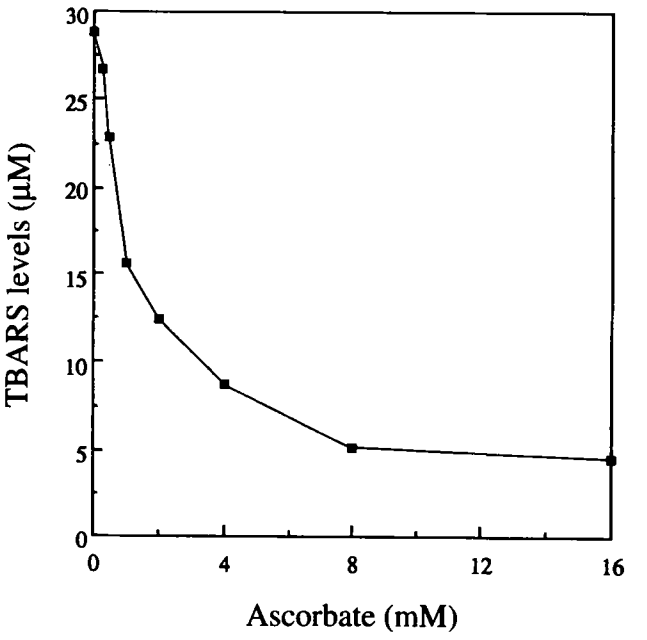


FIG. 3. Effect of added ascorbate on the levels of thiobarbituric acid-reactive substances (TBARS) in human blood plasma treated with 2,2'-azo*bis*(2-amidinopropane)HCl (AAPH). To tubes containing 50 μL of plasma and 60 μL of phosphate-buffered saline were added ascorbate to give the designated final concentrations. AAPH concentration was 30 mM. Tubes were incubated at 37°C for 4.5 h, and levels of TBARS were determined as described in the Experimental Procedures section. Values represent the average of quadruplicate determinations. The TBARS level in untreated control samples was 4.12 μM.

ascorbate to block AAPH-induced reduction of LCAT activity was tested at lower concentrations of the antioxidant.

Added ascorbate in the concentration range of 0.030–0.960 mM was able to partially prevent reduction of LCAT activity in plasma incubated with 30 mM AAPH to induce lipid peroxidation (Fig. 4). The protective effect of added ascorbate was concentration-dependent up to 0.480 mM. In the absence of added ascorbate, a 72% decrease in LCAT activity was found. However, the presence of 0.030, 0.060, 0.120, 0.240, and 0.480 mM added ascorbate allowed only 67, 58, 42, 34, and 28% decreases in LCAT activity, respectively. Higher levels of added ascorbate up to 2 mM (data not shown) were unable to block AAPH-induced reduction of LCAT activity to any greater extent than did 0.48 mM.

DISCUSSION

AAPH was used to induce oxidant stress in plasma. When incubated at 37°C, this thermally labile compound generates peroxyl radicals at a constant rate (11), and a time-dependent reduction of LCAT activity occurred (Fig. 1). A sharp de-

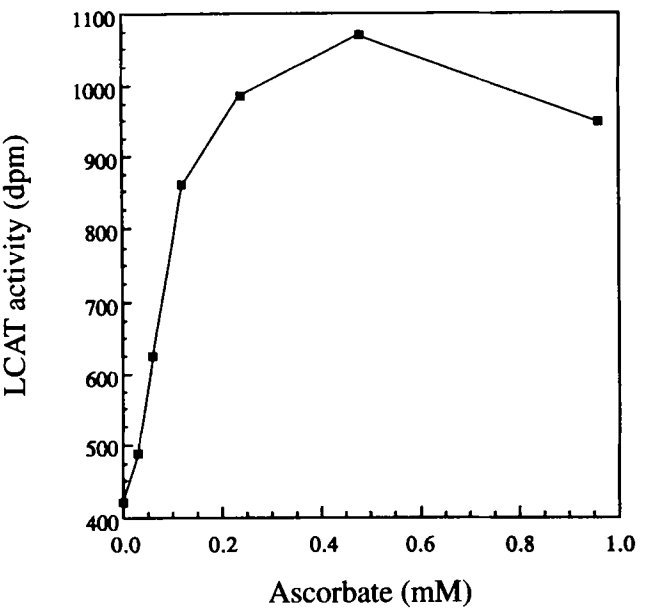


FIG. 4. Effect of added ascorbate on loss of lecithin/cholesterol acyltransferase (LCAT) activity in human blood plasma caused by 2,2'-azo*bis*(2-amidinopropane)HCl (AAPH). Conditions were as in Figure 3 except that at the start of equilibration of radiolabel during the LCAT assay, ascorbate was added to give the designated final concentrations. LCAT activity is expressed as amount of radioactivity detected in the cholesteryl ester fraction, and values are the average of two determinations. The control sample yielded 1495 dpm.

crease (30%) in LCAT activity was observed at the end of 15 min. This striking effect of AAPH might reflect rapid oxidative depletion of the β-mercaptoethanol that was added to initiate the enzyme reaction. On the other hand, it could be related to depletion of ascorbate and protein thiols which happens early when plasma is incubated with AAPH (12,13). Only slight additional reduction of LCAT activity was seen over the next 1.75 h. During this lag phase, other hydrophilic antioxidants such as bilirubin and urate might be depleted by AAPH-derived peroxyl radicals (12,13). Thereafter, however, LCAT activity decreased at a relatively faster rate. This might occur as α-tocopherol is simultaneously depleted after oxidation of all hydrophilic antioxidants (12,13).

Results in Figure 1 are consistent with recent work by McCall *et al.* (14). They observed a similar inhibition profile of LCAT activity when plasma was exposed to another form of oxidant stress, gas-phase cigarette smoke. A sharp decrease (56%) in LCAT activity occurred at the end of 1 h, followed by a lag phase of 2 h, and then noticeable resumption in the rate of decrease in LCAT activity for the next 3 h. Rapid depletion of endogenous plasma ascorbate occurred at the end of 1 h, parallelling the sharp reduction in LCAT activity. However, they also reported that addition of ascorbate at 85 μM concentration did not prevent reduction of LCAT activity. In contrast, reduced GSH significantly protected against cigarette smoke-induced inhibition of LCAT, suggesting that GSH preserved free functional sulfhydryl groups at the active site of LCAT.

Reduction of LCAT activity in AAPH-treated plasma did not appear to correlate closely with lipid peroxidation (Fig. 2). No inverse relationship can be established between reduction of LCAT activity and TBARS formation (Fig. 2). Ascorbate was able to prevent AAPH-induced lipid peroxidation (Fig. 3), as previously demonstrated (13), and also, to a large degree, the reduction of LCAT activity (Fig. 4) but the concentration-dependencies of these effects were not similar. Ascorbate at a concentration of 0.25 mM had little impact in preventing AAPH-induced formation of TBARS (Fig. 3), yet concentrations of ascorbate under 0.25 mM were able to prevent appreciable reduction of LCAT activity (Fig. 4). Lipid peroxidation can occur once the endogenous ascorbate in plasma has been depleted (12,13), but the capacity of ascorbate to protect against reduction of LCAT activity may not be due to its ability to block lipid peroxidation. More likely, ascorbate directly neutralizes AAPH-derived peroxyl radicals.

These above results suggest that LCAT activity can be down-modulated by oxidant stress, but not necessarily by lipid peroxidation. McCall and co-workers (14) have reached a similar conclusion, suggesting that greatest reduction of LCAT activity in plasma treated with cigarette smoke seems to precede lipid peroxidation. The mechanism by which AAPH causes reduction in LCAT activity remains to be elucidated. It is possible that peroxyl radicals generated from AAPH may directly attack LCAT, causing protein structural alteration. Reduction of LCAT activity might be the result of oxidation of cysteinyl free sulfhydryl groups at the active site of the enzyme. Recent studies by Frei *et al.* (12,13) have revealed that incubating human plasma, which had undergone prior gel filtration to remove endogenous ascorbate, with 50 mM AAPH leads to rapid depletion of protein thiols before the onset of lipid peroxidation. Over half the protein thiols in plasma were oxidized in less than 60 min of incubation. Added ascorbate (100 μM) was unable to prevent near complete depletion of protein thiols at the end of 180 min of incubation. Between 40 and 120 min, however, this concentration of ascorbate had a marginal but noticeable effect in retarding the rate of oxidation of protein thiols. It was not made known if higher concentrations of ascorbate could totally block AAPH-induced oxidation of protein thiols in plasma. It was suggested that protein thiols undergo autoxidation in scavenging AAPH-derived peroxyl radicals and that ascorbate cannot effectively negate this autoxidative process. Hence, in our study this could explain why added ascorbate was unable to completely prevent AAPH-induced loss of LCAT activity.

An additional explanation of the effect of AAPH may be that the peroxyl radicals produced from it may attack apolipoprotein A-I, which is a known activator of LCAT (15). This would reduce availability of intact protein cofactor to activate LCAT and decrease enzyme activity. Oxidative modification of HDL by cupric ions results in structural damage to apolipoprotein A-I and also in a modified HDL species less capable of promoting cholesterol efflux from macrophage-derived foam cells (3,4).

In conclusion, additional work is needed to uncover the mechanism by which oxidant stress reduces LCAT activity. However, it seems that reduction of LCAT activity in plasma subjected to AAPH-induced oxidant stress does not correlate directly with lipid peroxidation. Thus, the relationship between oxidant stress, reduction of plasma LCAT activity, and increased atherogenesis is in need of further study.

ACKNOWLEDGMENTS

This study was supported by The North Carolina Agricultural Research Service, The University of North Carolina Institute of Nutrition, and The University of North Carolina at Greensboro Research Council.

REFERENCES

1. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witztum, J.L. (1989) Beyond Cholesterol: Modifications of Low Density Lipoprotein That Increase Its Atherogenecity, *New Engl. J. Med. 320*, 915–924.
2. Bowry, V.W., Stanley, K.K., and Stocker, R. (1992) High Density Lipoprotein Is the Major Carrier of Lipid Hydroperoxides in Human Plasma from Fasting Donors, *Proc. Natl. Acad. Sci. USA 89*, 10316–10320.
3. Sakai, M., Miyazaki, A., Sakamoto, Y.-I., Shichiri, M., and Horiuchi, S. (1992) Cross-Linking of Apolipoproteins is Involved in a Loss of the Ligand Activity of High Density Lipoprotein upon Cu^{2+}-Mediated Oxidation, *FEBS Lett. 314*, 199–202.
4. La Ville, A.E., Sola, R., Balanya, J., Turner, P.R., and Masana, L. (1994) *In Vitro* Oxidised HDL Is Recognised by the Scav-

enger Receptor of Macrophages: Implications for Its Protective Role *in Vivo*, *Atherosclerosis 105*, 179–189.
5. Glomset, J.A. (1968) The Plasma Lecithin/Cholesterol Acyl-Transferase Reaction, *J. Lipid Res. 9*, 155–167.
6. Maziere, J.-C., Myara, I., Salmon, S., Auclair, M., Haigle, J., Santus, R., and Maziere, C. (1992) Copper- and Malondialdehyde-Induced Modification of High Density Lipoprotein and Parallel Loss of Lecithin/Cholesterol Acyltransferase Activation, *Atherosclerosis 104*, 213–219.
7. Wallentin, L., and Vikrot, O. (1975) Evaluation of an *in Vitro* Assay of Lecithin/Cholesterol Acyl Transfer Rate in Plasma, *Scand. J. Clin. Lab. Invest. 35*, 661–667.
8. Stokke, K.T., and Norum, K.R. (1971) Determination of Lecithin/Cholesterol Acyltransfer in Human Blood Plasma, *Scand. J. Clin. Lab. Invest. 27*, 21–27.
9. Buege, J.A., and Aust, S.D. (1978) Microsomal Lipid Peroxidation, *Methods Enzymol. 30*, 302–310.
10. Zannoni, V., Lynch, M., Goldstein, S., and Sato, P. (1974) A Rapid Micromethod for the Determination of Ascorbic Acid in Plasma and Tissues, *Biochem. Med. 11*, 41–48.
11. Niki, E. (1990) Free Radical Initiators as Source of Water- or Lipid-Soluble Peroxyl Radicals, *Methods Enzymol. 186*, 100–108.
12. Frei, B., Stocker, R., and Ames, B.N. (1988) Antioxidant Defenses and Lipid Peroxidation in Human Blood Plasma, *Proc. Natl. Acad. Sci. USA 85*, 9748–9752.
13. Frei, B., England, L., and Ames, B.N. (1989) Ascorbate Is an Outstanding Antioxidant in Human Blood Plasma, *Proc. Natl. Acad. Sci. USA 86*, 6377–6381.
14. McCall, M.R., van den Berg, J.J.M., Kuypers, F.A., Tribble, D.L., Krauss, R.M., Knoff, L.J., and Forte, T.M. (1994) Modification of LCAT Activity and HDL Structure: New Links Between Cigarette Smoke and Coronary Heart Disease Risk, *Arterioscler. Thromb. 14*, 248–253.
15. Fielding, C.J., Shore, V.G., and Fielding, P.E. (1972) A Protein Cofactor of Lecithin/Cholesterol Acyltransferase, *Biochem. Biophys. Res. Commun. 46*, 1493–1498.

[Received April 11, 1994, and in final revised form February 23, 1995; Revision accepted May 1, 1995]

Kinetic Analysis of Cardiolipin Synthase: A Membrane Enzyme with Two Glycerophospholipid Substrates

Michael Schlame[a,b], Ming Zhao[a], Diego Rua[a], Dipak Haldar[b] and Miriam L. Greenberg[a,*]

[a]Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202 and [b]Department of Biological Sciences, St. John's University, Jamaica, New York 11439

ABSTRACT: Mitochondrial cardiolipin synthase catalyzes the transfer of a phosphatidyl moiety from phosphatidyl-CMP (PtdCMP) to phosphatidylglycerol (PtdGro) in the presence of specific divalent cations. The synthase was solubilized from *Saccharomyces cerevisiae* mitochondria and purified about 300-fold. The partially purified enzyme was part of a medium-size, mixed micelle which had to bind to a foreign substrate/detergent micelle before catalysis could occur. The kinetics of cardiolipin synthase were studied by changing the molar fraction of substrate in the micelles. The enzyme obeyed Michaelis-Menten kinetics in relation to PtdCMP with a K_m of 0.03 mol%. PtdGro caused sigmoidal kinetics with a low apparent affinity. It is speculated that it was involved in docking the enzyme to the substrate/detergent micelle. Cardiolipin synthase did not catalyze isotope exchange between [^{14}C]CMP and PtdCMP, virtually excluding a ping-pong catalytic mechanism. Mg^{2+} stimulated the activity by increasing the turnover number rather than the substrate affinity, a mechanism which was also found for the Co^{2+}-activation of rat liver cardiolipin synthase. It is concluded that a direct association of the metal ion and the enzyme forms the active cardiolipin synthase which has a very high affinity for PtdCMP and a lower affinity for PtdGro.
Lipids 30, 633–640 (1995).

Cardiolipin is an essential phospholipid of mitochondria and some bacteria (1). It has a dimeric structure in which two phosphatidyl moieties are linked by a glycerol bridge; thus, its synthesis requires two phospholipid molecules as precursors. There is a eukaryotic and a prokaryotic reaction for cardiolipin biosynthesis, the former being a phosphatidyl transfer from phosphatidyl-CMP (CDP-diacylglycerol) (PtdCMP) to phosphatidylglycerol (PtdGro) (2–4), the latter being a phosphatidyl transfer between two PtdGro's (1). Cardiolipin formation in eukaryotes is dependent on the presence of divalent metal cations, such as Mg^{2+}, Mn^{2+}, or Co^{2+} (3–5), and is inhibited by Ba^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+} (5), as well as the polyvalent cation ruthenium red (6). Cardiolipin synthases from individual organisms have different cation specificities, showing highest activity with either Co^{2+} (mammals, *Neurospora crassa*) or Mg^{2+} (yeast, higher plants, molluscs), respectively (4). It is not known which cation is the authentic activator of cardiolipin biosynthesis *in vivo*, although Mg^{2+} is a likely candidate because it was effective in all organisms studied (3–5) and it is sufficiently concentrated in the cell. Cardiolipin synthase was purified from rat liver to characterize its properties, in particular the high sensitivity to its lipid environment (7).

In as much as both substrates of cardiolipin synthase are glycerophospholipids, which are insoluble in water, the kinetics of this enzyme are of particular significance. Extensive studies of the kinetic behavior in nonsoluble lipid aggregates have been performed for several enzymes which use a single lipid substrate, such as phospholipase A_2 (8,9), diacylglycerol kinase (10), or glycerol-3-phosphate acyltransferase (11). Recently, such experiments have been extended to the yeast enzymes PtdCMP synthase (12), phosphatidylserine synthase, phosphatidylinositol synthase (13), and phosphatidylinositol 4-kinase (14). Micellar detergent/phospholipid mixtures were used in the kinetic studies since they provided the enzymes with a well-dispersed substrate at a defined lipid/water interface. Because it has two lipid substrates instead of one, cardiolipin synthase adds an additional dimension to the problem of interfacial kinetics. To study its properties in a micellar system, yeast cardiolipin synthase was solubilized by Triton X-100 (Pierce Chemical Company, Rockford, IL) and partially purified. The major goal of this work was to compare the function of the two substrate phospholipids and to elucidate the role of the divalent cation in the catalytic process.

EXPERIMENTAL PROCEDURES

Materials. Phenylmethylsulfonylfluoride (PMSF), leupeptin, and aprotinin were obtained from Boehringer (Mannheim, Germany). *N*-ethylmaleimide and pepstatin A were from Sigma (St. Louis, MO). Blue Sepharose CL-6B, Q-Sepharose Fast Flow, S-Sepharose Fast Flow, and the Superose 12 column (HR 10:30) were obtained from Pharmacia (Uppsala, Sweden). Precoated silica gel 60 plates were from Merck (Darmstadt, Germany), and DE-52 anion exchange resin was

*To whom correspondence should be addressed at Department of Biological Sciences, Wayne State University, 5047 Gullen Mall, Detroit, MI 48202.

Abbreviations: LIPID, total lipid concentration; NMR, nuclear magnetic resonance; PMSF, phenylmethylsulfonylfluoride; PtdCMP, phosphatidyl-CMP (CDP-diacylglycerol); PtdGro, phosphatidylglycerol.

Copyright © 1995 by AOCS Press

from Whatman BioSystems Ltd. (Maidstone, United Kingdom). PtdGro (derived from egg yolk phosphatidylcholine) was obtained from Sigma and Triton X-100 was from Pierce Chemical Company. 1,2-Dimyristoyl-*sn*-[U-^{14}C]glycero-3-diphospho-5′-cytidine ([^{14}C]PtdCMP) was synthesized exactly as described (15). Its specific radioactivity was 88 dpm/pmol. 1,2-Dimyristoyl-*sn*-[2-^{3}H]glycero-3-phospho-5′-cytidine ([^{3}H]PtdCMP) was synthesized from [2-^{3}H]glycerol by the following procedure: (i) enzymatic phosphorylation to *sn*-glycero-3 phosphate by glycerokinase (16), (ii) chemical acylation to dimyristoylphosphatidic acid (15), and (iii) enzymatic conversion to PtdCMP by incubation with CTP and membranes of the PtdCMP synthase overproducing *Escherichia coli* strain DH1-pCD100 (3). The specific radioactivity of the [^{3}H]PtdCMP was 1070 dpm/pmol. [5-^{3}H]Cytidine-5′-monophosphate ([^{3}H]CMP) was obtained by acid hydrolysis of [5-^{3}H]cytidine-5′-triphosphate. A 50 mM solution of the nucleoside triphosphate was incubated for 8 h in 0.1 M Tris (pH 6) at 90°C. The degree of hydrolysis was monitored by thin-layer chromatography on silica gel G plates developed with 1-butanol/acetone/acetic acid/1.6% ammonia (45:15:10:30). The specific radioactivity of [^{3}H]CMP was 4.4 dpm/pmol.

Partial purification of cardiolipin synthase from yeast. The wild-type *Saccharomyces cerevisiae* strain D273-10B (*met6* MATa) was grown to mid-logarithmic phase at 30°C in a medium containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose. The harvested cells were stored at −80°C and rethawed for the enzyme preparation which was performed in a cold room (4°C). The cell paste (100 g wet weight) was washed with water and then suspended in 400 mL of medium containing 50 mM Tris (pH 7.5), 20% (vol/vol) glycerol, 0.5 mM PMSF, and 4 mM NaF. The cells were disrupted by homogenization with prechilled glass beads in a Biospec (Bartlesville, OK) Products Bead Beater. The homogenate was spun at 1000 × *g* for 5 min to remove glass beads and unbroken cells, and the supernatant was spun at 17,500 × *g* for 10 min to pellet crude mitochondria. The mitochondria were resuspended in 100 mL isolation medium, containing 50 mM Tris (pH 7.5), 0.3 M sucrose, 1 mM EDTA, and 0.5 mM PMSF, and centrifuged at 2000 × *g* for 5 min. Mitochondria remained in the supernatant from which they were pelleted by centrifugation at 17,500 × *g* for 15 min. The final mitochondrial pellet was resuspended in a small volume of isolation medium and then diluted by 100 mL of 5 mM phosphate buffer (pH 7.5) containing 0.25 mM EDTA, 0.25 mM EGTA, 0.5 mM PMSF, 0.1 mM *N*-ethylmaleimide, 1 mg/L pepstatin A, 1 mg/L leupeptin, and 2 mg/L aprotinin. This suspension was gently stirred for 20 min in order to swell the mitochondria. After that, the pH was adjusted to 8.5 with several drops of a 1 M Tris solution and solid KSCN (potassium thiocyanate; General Chemical Co., New York, NY), a chaotropic salt, was added to a final concentration of 1M. Stirring was continued for 30 min, and the suspension was sonicated for 1 min using a Model W140 Sonifier Cell Disruptor (Ultrasonics, Inc., Plainview, NY) set at 70% of maximal power output. The KSCN-extracted mitochondrial membranes were pelleted by ultracentrifugation (100,000 × *g*; 1 h) and resuspended in about 4 mL of medium which contained 30 mM Tris (pH 7.5; 4°C), 20% (vol/vol) glycerol, 0.5 mM EDTA, and 0.5 mM EGTA. The protein concentration of the membranes was adjusted to 10 g/L, Triton X-100 was added to a final concentration of 2% (wt/vol), and solid KCl was added to a final concentration of 0.05 M. The suspension was incubated for 30 min after which it was homogenized in a Dounce tissue grinder (Wheaton Dounce Tissue Grinders, Millville, NJ). Nonsolubilized membranes were spun down at 25,000 × *g* for 15 min and the supernatant was used for further preparations. During solubilization and all chromatographic procedures, the following cocktail of protease inhibitors was present: 0.5 mM PMSF, 0.1 mM *N*-ethylmaleimide, 1 mg/L pepstatin A, 1 mg/L leupeptin, and 2 mg/L aprotinin. All inhibitors were freshly added to the solvents prior to use. The Triton-extract was consecutively passed through a DEAE-anion exchange column (DE-52), a Cibacron Blue 3G-A column (Blue Sepharose CL-6B), and a Q-Sepharose Fast Flow anion exchange column. The pass-through fraction was collected at each step. The columns were equilibrated with a medium containing 30 mM Tris (pH 7.5; 4°C), 20% (vol/vol) glycerol, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5% Triton X-100. Their bed volume was 60% of the volume of the Triton-extract. The pass-through fraction of the Q-Sepharose column was loaded on a S-Sepharose Fast Flow cation exchange column which had the same bed volume as the applied fraction and which was equilibrated in the same medium as described above. After loading the sample, the column was washed with one bed volume of equilibration medium. Subsequently, the enzyme was eluted by equilibration medium containing 1 M KCl. One milliliter fractions were collected and assayed for cardiolipin synthase activity. The peak fractions were pooled, concentrated, and applied on a Superose 12 HR 10:30 gel filtration column equilibrated with a medium containing 30 mM Tris (pH 7.5; 4°C), 20% (vol/vol) glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 0.2% (wt/vol) Triton X-100, and 1 M KCl. Fractions of 0.8 mL were collected and assayed for activity. The two peak fractions were pooled and used for the kinetic studies. The enzyme preparation did not contain detectable levels of phospholipid contamination (determined in the dried, ashed lipid extract by colorimetric measurement of the phosphomolybdate complex).

Isolation and permeabilization of rat liver mitochondria. Rat liver mitochondria were prepared by a standard differential centrifugation procedure at a temperature of 0–4°C. Sprague-Dawley rats were starved overnight and decapitated, and the liver was homogenized in 0.25 M sucrose containing 20 mM Tris (pH 7.4) and 0.5 mM EDTA. The homogenate was spun at 600 × *g* for 5 min, the supernatant was removed and spun at 6,000 × *g* for 4 min. The pellet was resuspended in the same buffer and centrifuged at 15,000 × *g* for 90 s. After an additional resuspension/centrifugation cycle, the pellet was homogenized in the isolation medium and stored at −20°C. Before the experiments, the mitochondrial suspension was rethawed and adjusted to a protein concentration of 25

g/L, followed by a 30-min preincubation with 1 mM *N*-ethylmaleimide and 4 mM Triton X-100 at 0°C.

Assay of cardiolipin synthesis. Cardiolipin synthase activity was assayed at 37°C by incubating the enzyme with radiolabeled PtdCMP and unlabeled PtdGro in a total volume of 0.11 mL (15). The phospholipids were added individually from 10-fold concentrated stock solutions which were prepared by sonication of the lipids in water. The reaction medium contained 50 mM Tris (pH 9.0), 0.25 mM EGTA, and Triton X-100. All ingredients were allowed to equilibrate for 5 min before the reaction was started by addition of a metal cation (either Mg^{2+} or Co^{2+}). The reaction was stopped by addition of 2 mL methanol. Extraction of lipids, chromatographic isolation of labeled cardiolipin, and scintillation counting were performed exactly as described previously (15). Specific assay conditions were as follows: To measure the enzyme in the purification procedure, samples were incubated for 1 h in the presence of 3.4 μM [^{14}C]PtdCMP, 1 mM PtdGro, 10 mM $MgCl_2$, and 0.8 mM Triton X-100. For the kinetic analysis of the partially purified yeast enzyme, 4 μg of protein were incubated for 40 min in the presence of various concentrations of radiolabeled PtdCMP, PtdGro, $MgCl_2$, and Triton X-100. The amount of Triton, which was added with the enzyme, was taken into consideration. [^{3}H]PtdCMP (1070 dpm/pmol) was used when working at low PtdCMP concentrations, and [^{14}C]PtdCMP (88 dpm/pmol) was used when higher concentrations (>1 μM) were required. Cardiolipin synthase activity in rat liver mitochondria was assayed for 1 h in the presence of 0.25 mg protein. Further details are given in the legends. Cardiolipin formation was linear with respect to time over the indicated incubation periods.

^{31}P nuclear magnetic resonance (NMR) spectroscopy. ^{31}P NMR measurements of PtdGro/Triton X-100 mixtures were performed under incubation conditions similar to those in the cardiolipin synthase assay. The medium contained 50 mM Tris (pH 9.0, 37°C), 0.25 mM EGTA, 10 mM $MgCl_2$, and 173 mM KCl. KCl was added because it was present in all cardiolipin synthase assays as a contaminant of the enzyme preparation. The total concentration of PtdGro plus Triton X-100 was 16.5 mM and different ratios of PtdGro/Triton X-100 were adjusted. The samples were placed in 10-mm probes and maintained at 37°C during aquisition of the NMR spectra. The spectra were obtained by averaging 400 scans recorded with a GN-300 NMR (300 MHz) (General Electric, Fremont, CA). The tip angle was 60°, the aquisition time was 0.54 s, the relaxation delay was 0.2 s, and the sweep window was 7518 Hz.

Other methods. The protein concentration was determined by the method of Lowry *et al.* (17). Nonlinear regression analysis was performed with the program Sigmaplot 4.0 (Jandel Scientific, San Rafael, CA) which uses the Marquardt-Levenberg algorithm.

RESULTS

Micellar properties of partially purified cardiolipin synthase from yeast. Cardiolipin synthase was solubilized by Triton X-100 from yeast mitochondrial membranes and partially purified as shown in Table 1. The specific activity of the final enzyme preparation suggested a purification of about 300-fold. Partially purified cardiolipin synthase was associated with Triton, as indicated by the following facts: (i) Relatively high concentrations of Triton X-100 were required to solubilize the enzyme, whereas the chaotropic salt KSCN was unable to release it from the membranes; and (ii) Decrease of Triton X-100 below its critical micellar concentration led to aggregation and irreversible inactivation of the enzyme (data not shown).

The size of a mixed protein/detergent micelle depends on the molecular mass of the protein and on the number of detergent binding sites on its surface. We determined the size of the enzyme/Triton complex by fast-pressure liquid chromatography gel filtration on a Superose 12 HR 10:30 column and compared it to the size of pure Triton X-100 micelles (Fig. 1). The size distribution of the cardiolipin synthase activity was virtually identical to that of pure Triton micelles with aggregation numbers 100 to 155 (18). Calibration of the gel filtration column showed that the apparent molecular mass of the cardiolipin synthase–Triton complex was about 70 kDa. Thus, the enzyme protein must be smaller than 70 kDa and the number of Triton molecules bound to the enzyme must be lower than 100.

Basic kinetic features of yeast cardiolipin synthase in a micellar system. The rate of cardiolipin formation was measured in a mixed micellar system containing Triton X-100, PtdCMP, and PtdGro. In this system, the total lipid concentration (LIPID) is defined as the sum of the bulk concentrations of Triton, PtdCMP, and PtdGro, while the molar ratio of the substrates is defined as [PtdCMP]/LIPID, and [PtdGro]/LIPID, respectively. As shown in Figure 2A, a proportional increase of the bulk concentrations of PtdGro and Triton X-100 resulted in a decrease in the reaction rate. Since the enzyme activity was not inhibited by Triton X-100 up to a concentration of 3 mM (3), detergent inactivation could be excluded as a possible cause. A more plausible explanation for the decreasing rate was the drop of the PtdCMP molar ratio resulting from the addition of PtdGro and detergent. This im-

TABLE 1
Partial Purification of Cardiolipin Synthase from *Saccharomyces cerevisiae*[a]

Purification step	Specific activity (nmol/h/mg protein)	Purification factor
Homogenate	0.10 ± 0.02	1
Mitochondria	0.25 ± 0.07	2
KSCN-extracted membranes	0.42 ± 0.19	4
Triton extract	0.52 ± 0.10	5
Q-Sepharose	2.18 ± 0.55	22
S-Sepharose	9.98 ± 5.77	100
Superose 12	30.79 ± 12.89	308

[a]The specific activities are average values (+ SEM) of three enzyme preparations; KSCN, a chaotropic salt; Q-Sepharose, S-Sepharose, and Seperose 12 (Pharmacia, Uppsala, Sweden); Triton extract (Pierce Chemical Company, Rockford, IL).

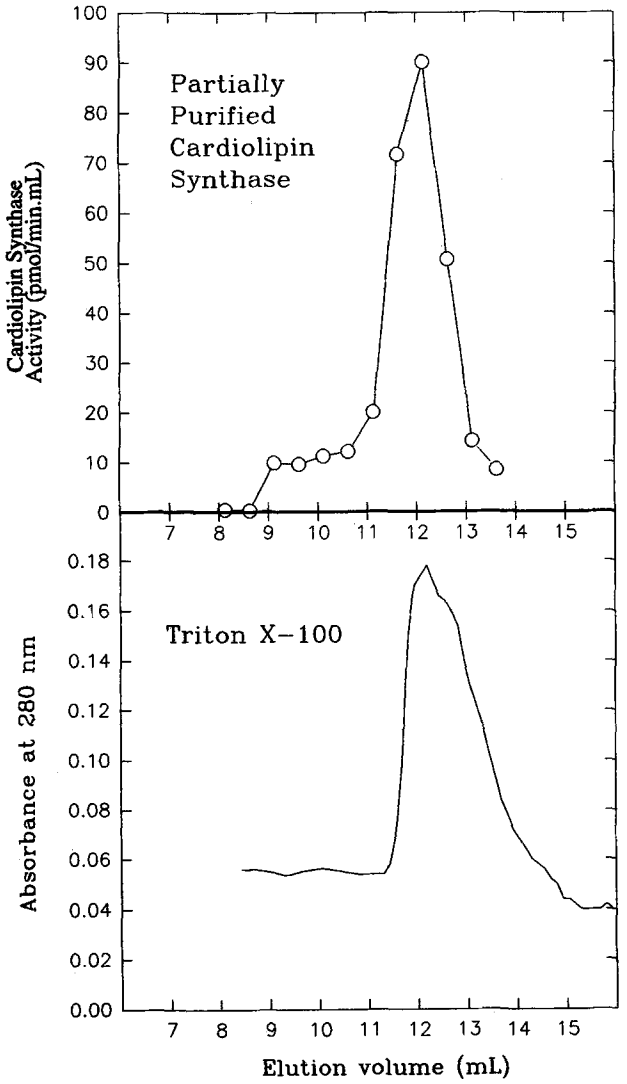


FIG. 1. Size-exclusion chromatography of partially purified cardiolipin synthase and Triton X-100 (Pierce Chemical Company, Rockford, IL) micelles. Upper panel: An aliquot of partially purified cardiolipin synthase was chromatographed on a Superose 12 HR 10:30 FPLC column (Pharmacia, Uppsala, Sweden). The column was equilibrated in 20% (vol/vol) glycerol, 0.03 M Tris (pH 7.5, 4°C), 0.2% (wt/vol) Triton X-100, 1 M KCl, 0.5 mM EDTA, and 0.5 mM EGTA, and it was run at a flow rate of 0.25 mL/min. Fractions of 0.5 mL were collected, and 25 µL aliquots were withdrawn for the determination of cardiolipin synthase activity. Lower panel: A sample of 1% (wt/vol) Triton X-100 was chromatographed under exactly the same conditions, and the elution of Triton was monitored spectrophotometrically at 280 nm.

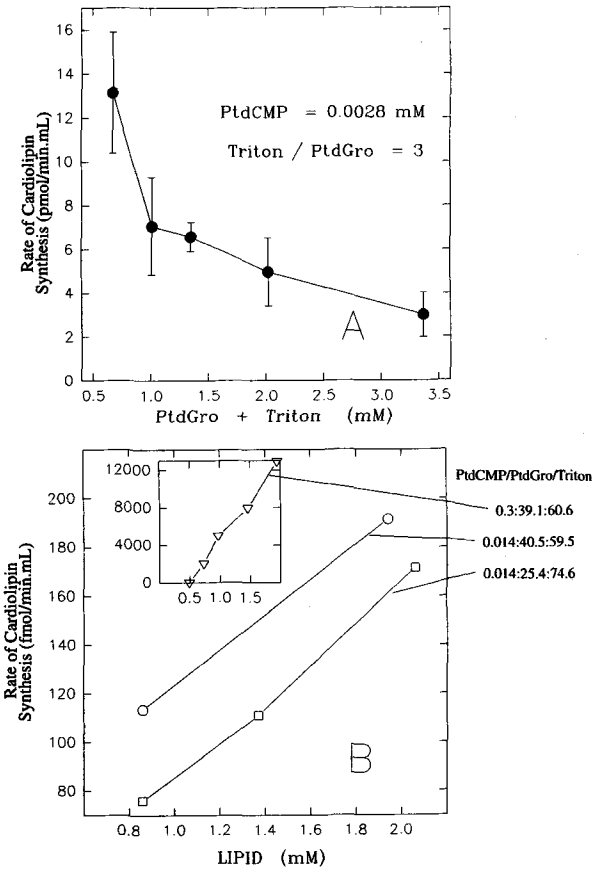


FIG. 2. Rate of cardiolipin synthesis at different micellar compositions. A: Dependence of the reaction rate on the concentration of phosphatidylglycerol (PtdGro) plus Triton X-100 at a fixed PtdGro/Triton ratio. The velocity of partially purified yeast cardiolipin synthase was measured in the presence of 2.8 µM phosphatidyl-CMP (CDP-diacylglycerol) (PtdCMP) and 10 mM Mg^{2+}. The absolute concentration of Triton and PtdGro was varied while maintaining a Triton/PtdGro ratio of 3. The data are mean values with standard error of mean (n = 3). B: Dependence of the reaction rate on the total lipid concentration at a constant molar ratio of lipids. The velocity of partially purified yeast cardiolipin synthase was measured at the indicated ratio of PtdCMP/PtdGro/Triton. The total concentration of lipids (LIPID = PtdCMP + PtdGro + Triton) was varied. The concentration of Mg^{2+} was 10 mM. The data are mean values of two experiments. Ranges were lower than 13%. See Figure 1 for company source.

plies that PtdCMP was presented to the enzyme as a PtdCMP/PtdGro/Triton aggregate and that it was the molar ratio of PtdCMP in this aggregate, rather than its bulk concentration that determined the rate of cardiolipin formation. When the total lipid concentration was increased while keeping the lipid ratio constant, the rate of cardiolipin formation increased as well (Fig. 2B). This experiment was repeated for different lipid ratios, demonstrating a dependence on that ratio at constant total lipid concentration: The rate was slightly higher in the presence of 40.5 mol% PtdGro than in the presence of 25.4 mol% PtdGro, and it increased about 50-fold when the PtdCMP ratio was enhanced from 0.014 to 0.3 mol% (Fig. 2B). In conclusion, the reaction rate was dependent on both the total lipid concentration and the molar ratios of the two substrates.

Kinetic dependence of yeast cardiolipin synthase on the molar fraction of PtdGro. The rate of cardiolipin formation was measured at constant total lipid concentration and changing molar fractions of PtdGro. The kinetics showed a sigmoidal behavior with an estimated Hill coefficient of 3.3 (Fig. 3). Substrate saturation was reached between 40 and 50 mol% of PtdGro; however, further increase of the PtdGro level led to strong inhibition of the cardiolipin formation. In the presence of low concentrations of cardiolipin, the sig-

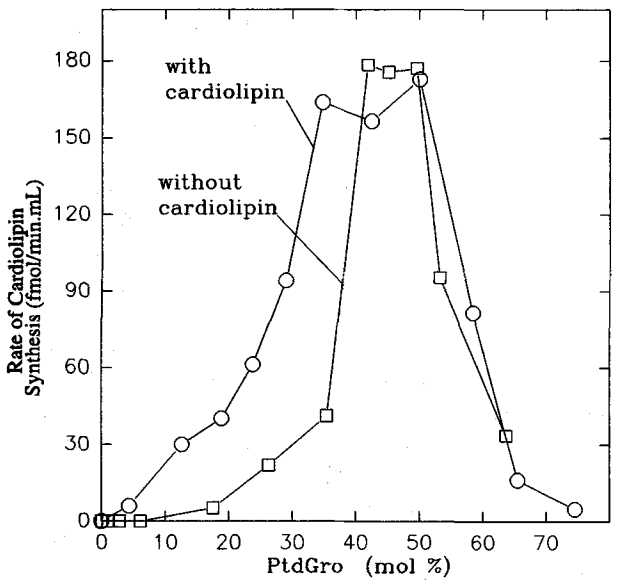


FIG. 3. Dependence of the reaction rate on the molar ratio of PtdGro in the presence and absence of cardiolipin. The velocity of partialy purified yeast cardiolipin synthase was measured at constant total lipid concentration [total lipid concentration (LIPID) = 1.7 mM] in the absence (□) and presence (○) of 3.2 mol% cardiolipin. The molar ratio of PtdGro was increased at expense of Triton X-100. For the calculation of the micellar PtdGro/LIPID ratio, the critical micellar concentration of Triton X-100 (0.25 mM) was subtracted from the total lipid concentration. The molar ratio of PtdCMP was 0.016 mol% and the concentration of Mg^{2+} was 10 mM. The data are mean values of two experiments. Ranges were lower than 28%. See Figure 2 for other abbreviations and company source.

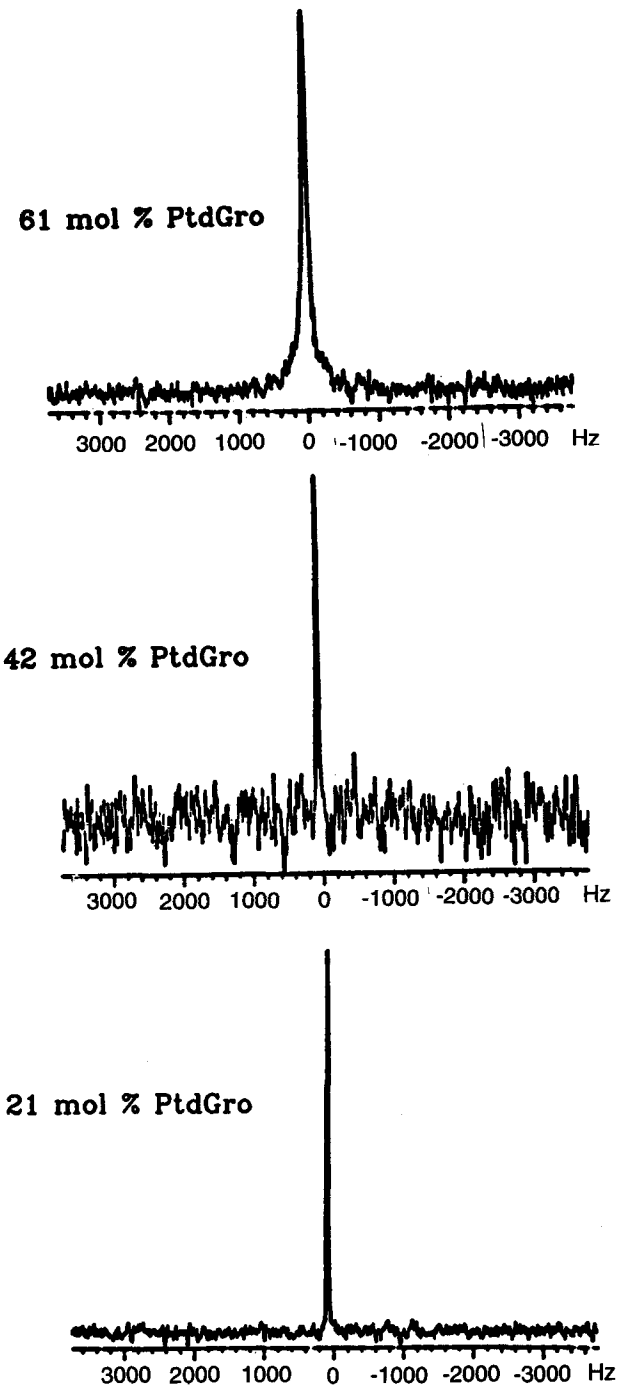


FIG. 4. ^{31}P nuclear magnetic resonance (NMR) spectra of different PtdGro/Triton X-100 mixtures. PtdGro and Triton X-100 were incubated in a medium similar to the cardiolipin synthase assay mixture. The total lipid concentration (LIPID = PtdGro + Triton) was 16.5 mM. ^{31}P NMR spectra were recorded for three different molar fractions of PtdGro. The improvement of the signal to noise ratio at higher PtdGro levels was partly offset by an increase of the sample viscosity. See Figure 2 for other abbreviations and company source.

moidal part of the curve was shifted toward lower PtdGro concentrations; thus, cardiolipin increased the apparent affinity of cardiolipin synthase for PtdGro. At the same time, it decreased the sigmoidality as reflected by a drop of the Hill coefficient from 3.3 to 1.5. In contrast, the neutral phospholipids phosphatidylcholine and phosphatidylethanolamine had no stimulating effect on cardiolipin formation (data not shown). Since the ratio of PtdGro to Triton X-100 was varied significantly in the kinetic experiment, it was important to have some information about the physical state of the lipids. To this end, we measured ^{31}P NMR spectra of PtdGro/Triton X-100 mixtures at the same ionic strength, pH, and Mg^{2+} concentration as in the assay system. The NMR sensitivity made it necessary to use higher absolute lipid concentrations than in the enzyme assay, but comparable lipid ratios were used (PtdCMP was omitted because it cannot affect the physical state due to its very low abundance). Figure 4 shows that, regardless of the PtdGro ratio (21, 42, and 61 mol%), a single, isotropical ^{31}P peak was found in all NMR spectra. This strongly suggested a micellar state since phase changes toward more lamellar structures were expected to result in the appearance of either asymmetrical NMR line shapes or two-component spectra (19). The NMR data were in line with a similar study on phosphatidylcholine/Triton X-100 mixtures, in which a purely isotropic state was preserved up to a lipid/detergent ratio of 1 (19). However, one must remember that ^{31}P NMR reports phase transitions only and is not susceptible to more subtle changes of the micellar structure.

Effect of Mg^{2+} on yeast cardiolipin synthase. The Mg^{2+} dependence of the purified yeast cardiolipin synthase is

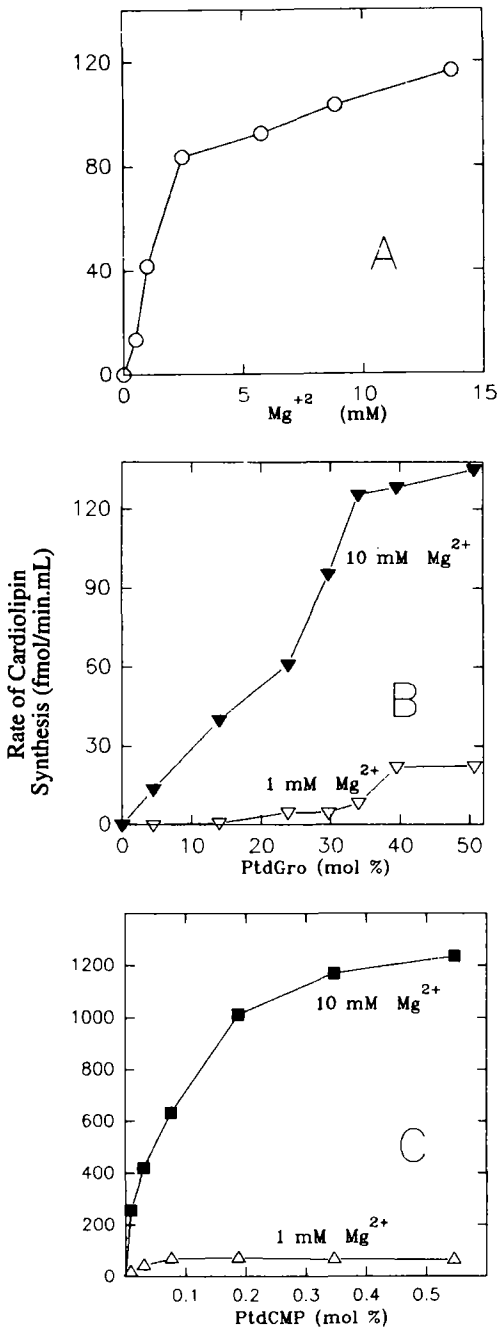


FIG. 5. The effect of Mg^{2+} on yeast cardiolipin synthase. A: The velocity of partially purified yeast cardiolipin synthesis was measured at different Mg^{2+} concentrations in the presence of 0.17 μM PtdCMP, 0.57 mM PtdGro, 1.0 mM Triton X-100, and 0.05 mM cardiolipin. B: The PtdGro dependence of cardiolipin synthesis was measured at low and high Mg^{2+} concentration. LIPID = 1.72 mM; PtdCMP = 0.17 μM; cardiolipin = 0.05 mM. The concentration of PtdGro was increased at expense of Triton X-100. For the calculation of the micellar PtdGro/LIPID ratio, the critical micellar concentration of Triton X-100 (0.25 mM) was subtracted from the total lipid concentration. C: The PtdCMP dependence of cardiolipin synthesis was measured at low and high Mg^{2+} concentration. LIPID = 1.72 mM; PtdGro = 0.46 mM; cardiolipin = 0.05 mM. For the calculation of the micellar PtdCMP/LIPID ratio, the critical micellar concentration of Triton X-100 (0.25 mM) was subtracted from the total lipid concentration. All data are average values of three experiments. Standard errors were lower than 29% of the respective mean values. See Figures 2 and 3 for abbreviations. See Figure 2 for company source.

shown in Figure 5A. The Mg^{2+} concentration required for half-maximal activity was 1.7 ± 0.7 mM as estimated by nonlinear fitting of the hyperbolic saturation curve. To investigate the Mg^{2+} effect in more detail, the substrate dependence of cardiolipin synthase was measured at optimal and suboptimal Mg^{2+} concentrations (Fig. 5B,C). Mg^{2+} affected both the PtdGro and the PtdCMP kinetics by increasing the maximal enzyme activity. However, there was no significant effect on the PtdCMP substrate affinity: The two K_m values for PtdCMP, each estimated by nonlinear regression analysis of 18 kinetic points, were 0.02 ± 0.01 and 0.03 ± 0.01 mol% in the presence of 1 and 10 mM Mg^{2+}, respectively. On the other hand, the apparent affinity to PtdGro was slightly higher at the higher Mg^{2+} concentration (Fig. 5B). It is important to note that, in contrast to PtdGro, the rate dependence on PtdCMP followed hyperbolic saturation kinetics (Fig. 5C). Thus, it was possible to estimate the apparent K_m value of PtdCMP but not that of PtdGro.

Measurement of isotope exchange between CMP and PtdCMP. PtdCMP contains an anhydride bond which is cleaved during the reaction with PtdGro. Thus, cardiolipin synthesis could potentially involve binding of PtdCMP as the first substrate followed by its conversion to a high-energy enzyme ~ Ptd intermediate and free CMP (ping-pong mechanism). If this were the case, cardiolipin synthase would catalyze isotope exchange between CMP and PtdCMP. To test this hypothesis, we measured the ^3H transfer from [^{3}H]CMP to PtdCMP in the presence of partially purified yeast cardiolipin synthase. The data of Table 2 show unequivocally that there was no enzyme-catalyzed exchange reaction between CMP and PtdCMP, even under conditions where the labeled substrate was in 30-fold excess over the unlabeled one. Addition of PtdGro to the reaction mixture did not stimulate the exchange reaction.

Effect of Co^{2+} on cardiolipin synthase in rat liver mitochondria. Co^{2+} is the most efficient cation for the activation

TABLE 2
Yeast Cardiolipin Synthase Does Not Catalyze an Exchange Reaction Between [^{3}H]CMP and PtdCMP[a]

	Recovery of [^{3}H]PtdCMP (pmol)		
Metabolites	25 min	75 min	180 min
300 nmol [^{3}H]CMP (10 nmol PtdCMP)	−1 ± 6	−3 ± 9	+5 ± 5
300 nmol [^{3}H]CMP (10 nmol PtdCMP + 30 nmol PtdGro)	−11 ± 6	+3 ± 2	+1 ± 3

[a]The indicated metabolites were dissolved in a total volume of 0.12 mL reaction buffer containing 50 mM Tris (pH 9.0), 0.25 mM EGTA, 1.3 mM Triton X-100, 10 mM $MgCl_2$, and 11 μg partially purified cardiolipin synthase. Samples were incubated at 37°C for 0, 25, 75, or 180 min. The incubation was stopped by lipid extraction followed by chromatographic isolation of phosphatidyl-CMP (CDP-diacylglycerol) (PtdCMP) on precoated silica gel 60 plates using the solvent chloroform/methanol/water/30% ammonia (70:38:2:8). The recovery of [^{3}H]PtdCMP was calculated from the radioactivity found in the PtdCMP fraction after subtracting the blank value (0 min incubation). Data are means (± SEM) of three experiments. See Table 1 for company source; PtdGro, phosphatidylglycerol.

of rat liver cardiolipin synthase (4,5), reaching saturation at about 5 mM (5,7). To characterize the effect of Co^{2+}, apparent kinetic parameters were determined at high (5 mM) and low (0.5 mM) Co^{2+} concentration in rat liver mitochondria. The kinetic curves obtained were hyperbolic, and kinetic parameters were derived by nonlinear fitting (Sigmaplot 4.0) of at least 8 data points for each parameter respectively. Co^{2+} activated cardiolipin formation by increasing the maximal enzyme activity from 2.9 ± 0.1 pmol/min/mg protein (at low Co^{2+}) to 4.1 ± 0.6 pmol/min/mg protein (at high Co^{2+}). The apparent K_m for PtdGro decreased from 61 ± 9 μM (at low Co^{2+}) to 32 ± 18 μM (at high Co^{2+}), while the apparent K_m for PtdCMP was not affected (2.4 ± 1.0 μM and 2.9 ± 1.6 μM at low and high Co^{2+}, respectively). Thus, the Co^{2+} effect on the liver enzyme was similar to that of Mg^{2+} on the yeast enzyme. This was true despite the differences in assay conditions between whole mitochondria (rat enzyme) and mixed micelles (yeast enzyme).

DISCUSSION

Cardiolipin synthase is a membrane-bound enzyme which uses two phospholipids as substrate. The enzyme was solubilized by Triton X-100, partially purified, and then used for kinetic experiments. In theory, there are three ways in which the solubilized cardiolipin synthase could interact with the substrate lipids: (i) The enzyme could react with soluble substrate monomers, (ii) the enzyme could react with substrate molecules associated with its own lipid/detergent anulus, and (iii) the enzyme could bind to a Triton/phospholipid micelle and react with substrates from this "foreign" micelle. In case (i), the reaction rate is expected to obey Michaelis-Menten kinetics with respect to the bulk concentrations of substrates. In case (ii), the rate should depend on the relative number of substrate molecules in the lipid/detergent anulus of the enzyme. Provided that the intermicellar lipid exchange is much faster than the reaction rate [Intermicellar phospholipid exchange may occur by both monomer diffusion and transient micelle collision. The rate constants of these processes were estimated for phosphatidylcholine/taurocholate mixed micelles (21,22). The data suggest that intermicellar lipid exchange is faster by several orders of magnitude than the maximal rate of cardiolipin formation measured ($2 \cdot 10^{-10}$ M^{-1} s^{-1}). Although the taurocholate/phosphatidylcholine system is different from the PtdCMP/PtdGro/Triton mixture, micellar exchange rates are expected to have the same order of magnitude.], this number is expected to be proportional to the molar fraction of substrates in the lipids [(PtdCMP)/LIPID and (PtdGro)/LIPID]. Case (iii) is analogous to a kinetic model developed for venom phospholipase A_2 (8,9). It predicts that the reaction rate depends on both the concentration of micelles (total lipid concentration) and the molar ratio of substrates in the micelles. This is what we found in the kinetic experiments with partially purified cardiolipin synthase (Figs. 2,3,5). Thus, it seems that cardiolipin synthase must first associate with the PtdCMP/PtdGro/Triton micelles before it can catalyze the formation of cardiolipin. However, it remains unknown whether the association between the enzyme/Triton micelle and the phospholipid/Triton micelle is achieved by surface attachment or by micelle fusion.

Most kinetic experiments were done at constant total lipid concentration and variable molar proportions of the lipid components. The rationale of that approach was to keep the overall surface area of the micelles approximately constant while changing the surface concentration of individual lipids (8). The fact that cardiolipin synthase requires high molar fractions of PtdGro, in order to be active, makes this assumption somewhat problematic, since structural changes of the micelle are likely to occur as PtdGro content increases from 0 to 70 mol%. The same does not apply to PtdCMP whose molar proportion was never above 0.5 mol%. Thus, at the present stage, there are several interpretations possible for the curves in Figure 3. For instance, the sigmoidal part of the PtdGro dependence may indicate that cardiolipin synthase interacts with more than one PtdGro molecule at a time. In addition to being a substrate, PtdGro may be an enzyme activator or may facilitate enzyme binding to the micelle. However, the fact that cardiolipin formation decreased when the PtdGro ratio was over 50 mol% is most likely due to changes of the micellar structure. While the NMR data do not suggest the formation of a lamellar-type phase (Fig. 4), other structural changes within the micellar phase cannot be excluded by NMR spectroscopy. In any case, PtdGro was crucial in forming the specific microenvironment of the enzyme. Cardiolipin, but not the neutral lipids phosphatidylcholine and phosphatidylethanolamine, was able to shift the PtdGro dependence toward lower concentrations (Fig. 3), suggesting that cardiolipin synthase was susceptible to both PtdGro and cardiolipin (and perhaps other acidic phospholipids).

Since a ping-pong catalytic mechanism seems to be very unlikely for cardiolipin synthase (Table 2), the enzyme is expected to form a ternary complex with the two substrate phospholipids. However, the kinetic data showed that the two phospholipids have different functional properties. PtdCMP shows hyperbolic kinetics with an extremely low K_m value (Fig. 5); thus, it behaves like a regular substrate with very high binding affinity. PtdGro, on the other hand, is not only a substrate but is also involved in establishing the proper environment for the enzyme (see above). As few as 2–3 molecules of PtdCMP in 10,000 lipid molecules are required for half-maximal activity of cardiolipin synthase while the equivalent PtdGro level is several hundredfold higher. Mitochondria contain PtdGro as a minor phospholipid, accounting for 1 to 10% of lipid phosphorus (22). The mitochondrial content of PtdCMP is usually below the detectable level, which provides a potential reason for the high PtdCMP affinity.

Divalent metal cations are specific cofactors of cardiolipin formation (3–5). In theory, these ions may either bind to the anionic substrates, forming lipid-metal complexes, or to the enzyme protein. If one of the authentic substrate species was, in fact, a metal complex, the K_m value of that substrate should depend on the metal ion concentration. We measured the sub-

strate affinities of cardiolipin synthases from yeast and rat liver at optimal and suboptimal concentrations of Mg^{2+} and Co^{2+}, respectively, and we found no differences in the PtdCMP affinity and only small differences in the PtdGro affinity. Instead, both metal ions had a significant effect on the maximal velocity; thus, it seems that divalent metal ions bind to the enzyme directly and become part of the catalytic site and/or induce a more active protein conformation. Therefore, the cation specificity of cardiolipin synthesis in different eukaryotes (4) reflects intrinsic properties of the respective enzymes rather than differences in the mitochondrial membrane composition.

ACKNOWLEDGMENTS

This work was supported by grant GM 37723 from the National Institute of Health and by grant DCB-8801535 from the National Science Foundation.

REFERENCES

1. Hostetler, K.Y. (1982) in *Phospholipids* (Hawthorne, J.N., and Ansell, G.B., eds.), pp. 215–261, Elsevier Biomedical Press, Amsterdam.
2. Hostetler, K.Y., van den Bosch, H., and van Deenen, L.L.M. (1971) Biosynthesis of Cardiolipin in Liver Mitochondria, *Biochim. Biophys. Acta 239*, 113–119.
3. Tamai, K.T., and Greenberg, M.L. (1990) Biochemical Characterization and Regulation of Cardiolipin Synthase in *Saccharomyces cerevisiae*, *Biochim. Biophys. Acta 1046*, 214–222.
4. Schlame, M., Brody, S., and Hostetler, K.Y. (1993) Mitochondrial Cardiolipin in Diverse Eukaryotes, *Eur. J. Biochem. 212*, 727–735.
5. Hostetler, K.Y., Galesloot, J.M., Boer, P., and van den Bosch, H. (1975) Further Studies on the Formation of Cardiolipin and Phosphatidylglycerol in Rat Liver Mitochondria, *Biochim. Biophys. Acta 380*, 382–389.
6. Schlame, M., and Haldar, D. (1993) Cardiolipin is Synthesized on the Matrix Side of the Inner Membrane in Rat Liver Mitochondria, *J. Biol. Chem. 268*, 74–79.
7. Schlame, M., and Hostetler, K.Y. (1991) Solubilization, Purification, and Characterization of Cardiolipin Synthase from Rat Liver Mitochondria, *J. Biol. Chem. 266*, 22398–22403.
8. Deems, R.A., Eaton, B.R., and Dennis, E.A. (1975) Kinetics Analysis of Phospholipase A_2 Activity Toward Mixed Micelles and Its Implications for the Study of Lipolytic Enzymes, *J. Biol. Chem. 250*, 9013–9020.
9. Hendrickson, H.S., and Dennis, E.A. (1984) Kinetic Analysis of the Dual Phospholipid Model for Phospholipase A_2 Action, *J. Biol. Chem. 259*, 5734–5739.
10. Walsh, J.P., and Bell, R.M. (1986) *sn*-1,2-Diacylglycerol Kinase of *Escherichia coli*, *J. Biol. Chem. 261*, 6239–6247.
11. Scheideler, M.A., and Bell, R.M. (1991) Characterization of the Active and Latent Forms of the Membrane-Associated *sn*-Glycerol-3-Phosphate Acyltransferase of *Escherichia coli*, *J. Biol. Chem. 266*, 14321–14327.
12. Kelley, M.J., and Carman, G.M. (1987) Purification and Characterization of CDP-Diacylglycerol Synthase from *Saccharomyces cerevisiae*, *J. Biol. Chem. 262*, 14563–14570.
13. Bae-Lee, M., and Carman, G.M. (1990) Regulation of Yeast Phosphatidylserine and Phosphatidylinositol Synthase Activities by Phospholipids in Triton X-100/Phospholipid Mixed Micelles, *J. Biol. Chem. 265*, 7221–7226.
14. Buxeda, R.J., Nickels, Jr., J.T., Belunis, C.J., and Carman, G.M. (1991) Phosphatidylinositol 4-Kinase from *Saccharomyces cerevisiae*, *J. Biol. Chem. 266*, 13859–13865.
15. Schlame, M., and Hostetler, K.Y. (1992) Mammalian Cardiolipin Biosynthesis, *Methods Enzymol. 209*, 330–337.
16. Hajra, A.K. (1968). Biosynthesis of Acyl Dihydroxyacetone Phosphate in Guinea Pig Liver Mitochondria, *J. Biol. Chem. 243*, 3458–3465.
17. Lowry, O.R., Rosenbrough, N.J., Farr, A.L., and Randall, A.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
18. Neugebauer, J. (1990) *A Guide to the Properties and Uses of Detergents in Biology and Biochemistry*, 3rd edn., p. 27, Calbiochem Corporation, La Jolla, CA.
19. Goñi, F.M., Urbaneja, M.-A., Arrando, J.L., Alonso, A., Durrani, A.A., and Chapman, D. (1986) The Interaction of Phosphatidylcholine Bilayers with Triton X-100, *Eur. J. Biochem. 160*, 659–665.
20. Nichols, J.W. (1988) Phospholipid Transfer Between Phosphatidylcholine-Taurocholate Mixed Micelles, *Biochemistry 27*, 3925–3931.
21. Fullington, D.A., and Nichols, J.W. (1993) Kinetic Analysis of Phospholipid Exchange Between Phosphatidylcholine/Taurocholate Mixed Micelles: Effect of the Acyl Chain Moiety of the Micellar Phosphatidylcholine, *Biochemistry 32*, 12678–12684.
22. Daum, G. (1985) Lipids of Mitochondria, *Biochim. Biophys. Acta. 822*, 1–42.

[Received August 30, 1994, and in revised form March 24, 1995; Revision accepted April 27, 1995]

Synergistic Induction of Phospholipid Metabolism by Granulocyte-Macrophage Colony Stimulating Factor and Steel Factor in Human Growth Factor-Dependent Cell Line, M07e

C. Mantel*, Z. Luo and H.E. Broxmeyer

Departments of Medicine (Hematology/Oncology) and Microbiology/Immunology, and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202-5121

ABSTRACT: Steel factor (SLF), the ligand for the c-kit proto-oncogene tyrosine kinase receptor, synergizes with several hematopoietic growth factors to produce greatly enhanced proliferation of normal human hematopoietic progenitor cells as well as that of the human growth factor-dependent myeloid cell line, M07e. The mechanisms of this phenomenon remain unknown. In an attempt to understand the cellular processes relevant to this phenomenon, we examined the effects of SLF and granulocyte-macrophage colony-stimulating factor (GM–CSF) on induced lipid metabolism in M07e cells. We find that both GM–CSF and SLF induced increased phosphatidylcholine (PC) turnover rates (biosynthesis and degradation) as measured by increased [^{3}H]-choline labelling, with SLF being more potent than GM–CSF after 6 h of stimulation, but equipotent at 24 h of stimulation. The labelling of aqueous intermediates of PC metabolism was also increased by cytokine stimulation, most notably phosphocholine. Simultaneous stimulation with GM–CSF plus SLF resulted in a true synergistic induction of PC, lysoPC, and phosphocholine labelling. GM–CSF and SLF each induced asymmetric labelling of various phospholipid classes as measured by incorporation of different [^{3}H]-fatty acids. [^{3}H]-myristic acid labelling of phosphatidylserine was most prominently induced (approximately 12-fold). Cytosolic choline kinase activity was also upregulated more than twofold over control by SLF, which might contribute to the increased phosphocholine labelling. These effects may have relevance to the intracellular mechanisms of the synergistic proliferative stimulation of SLF plus GM–CSF on M07e cells.

Lipids 30, 641–647 (1995).

In recent years the study of phospholipid metabolism has achieved a new aspect of interest due mainly to the findings of the roles that phospholipids (PLs) play in cellular signal transduction. The hydrolytic cleavage of phosphatidylinositol (PI), phosphatidylcholine (PC), and sphingomyelin (SPH) by various phospholipases has been strongly implicated in a number of cell surface receptor mediated signal transduction processes (1–3). More recently, the biosynthesis of PC, the major eukaryotic membrane PL, has been shown to be closely coordinated with events in the cell cycle (4) that can be regulated by extracellular hormones, including the cytokines that regulate blood cell production. One of these cytokines is granulocyte-macrophage colony-stimulating factor (GM–CSF). It regulates the proliferation and differentiation of hematopoietic progenitor cells leading to the eventual generation of myeloid blood cells (i.e., granulocytes, monocytes) (5,6). The GM–CSF receptor is a heterodimeric transmembrane protein that contains no known intrinsic catalytic activity, but its activation results in rapid tyrosine and serine/threonine phosphorylation of many proteins (7,8), the activation of PI hydrolysis by phospholipase C (9), and the activation of transcription factors (10) ultimately culminating in mitosis in established myeloid cell lines.

Another cytokine is steel factor (SLF), the ligand for the proto-oncogene encoded c-kit receptor protein. The SLF-receptor is a homodimeric transmembrane protein with intrinsic tyrosine kinase activity (11), the activation of which results in signals, some of which are the same, and some different from those of the GM–CSF receptor (7,8,12) in the growth factor-dependent human myeloid cell line M07e. Simultaneous stimulation of this cell line with GM–CSF and SLF results in an interesting phenomenon known as synergistic proliferation (i.e., greater-than-additive effect) (7,8,12). This phenomenon has not yet been adequately explained by modulation of receptor number or affinity, or by effects on signal transduction events such as tyrosine phosphorylation. However, we have previously reported a relationship of immediate early gene transcription (10), as well as choline-lipid metabolism (13), to synergistic stimulation in M07e cells. In the latter study (13) we found that GM–CSF and SLF upregulated PC biosynthesis and degradation (i.e., turnover) and that co-stimulation with both cytokines resulted in a much greater effect, which was temporally associated with synergistic cell proliferation. Since GM–CSF and SLF both are known to activate phospholipases

*To whom correspondence should be addressed at Walther Oncology Center, Indiana University School of Medicine, 975 West Walnut St., Room 501, Indianapolis, IN 46202-5121.

Abbreviations: AA, arachidonic acid; CK, choline kinase; DAG, diacylglycerol; FA, fatty acid; GM-CSF, granulocyte-macrophage colony stimulating factor; LPC, lysophosphatidylcholine; MA, myristic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PKC, protein kinase-C; PL, phospholipid; PS, phosphatidylserine; SLF, steel factor; SPH, sphingomyelin.

Copyright © 1995 by AOCS Press

as part of their signal transduction pathways, we had proposed that the cooperative increase in choline–lipid metabolism may be relevant to synergistic growth signals. We also suggested that these cytokines might upregulate the activity of choline kinase, one of the enzymes of PC biosynthetic pathways in M07e cells (13). This was based on the observation of the accumulation of label in the phosphocholine pool while label decreased in the choline pool.

This line of investigation has now been extended by analyzing lipid metabolism at different time points after stimulation and by examining effects of these two cytokines on different lipid classes labelled with different fatty acids (FAs). The results demonstrate that a bona fide synergistic response to GM–CSF plus SLF in PC turnover occurs in M07e cells, and that growth-factor induced PL metabolism in M07e cells is PL class- and species-specific. Additionally, choline kinase activity is increased by these cytokines.

METHODS AND MATERIALS

Materials. M07e cells are a cell line originally isolated from a patient with acute megakaryocytic leukemia, which is dependent for growth on stimulation by cytokines such as GM–CSF, interleukin-3, and SLF (14). Their growth characteristics, and conditions for "factor-starvation," have been previously characterized (7,14). Culture conditions were exactly as described elsewhere (14). Purified recombinant human (rhu) SLF and rhu GM–CSF were gifts from Dr. D.E. Williams (Immunex Corp., Seattle, WA). Silica Gel 60A thin-layer chromatography plates were from Whatman International (Maidstone, England). [^{3}H]-choline, [^{3}H]-myristic acid (MA), and [^{3}H]-arachidonic acid (AA) were from Amersham Corp. (Arlington Heights, IL). Lipid standards and all other reagents were from Sigma Chemical Co. (St. Louis, MO). Protein was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Labelling of cells and analysis of labelled cellular lipids. Cell labelling and choline lipid separation and analysis were done as previously described (13). For some experiments, cells were labelled in a similar fashion, except with [^{3}H]-MA or [^{3}H]-AA (1 μCi/mL). The separation of [^{3}H]-FA labelled cellular PL classes was done by two-dimensional thin-layer chromatography as described previously (15). PL spots were visualized and quantitated as above for [^{3}H]-choline labelled lipids.

Cell-free choline kinase (CK) assay. The *in vitro* CK activity of whole cell cytosolic homogenates was measured essentially as described by others (16), except [^{3}H]-choline was used instead of [^{14}C]-choline, and the reaction was terminated by adding 3 mL of chloroform/methanol (1:2, vol/vol). Extraction and separation of phosphocholine was done the same as for cellular lipids (described previously). The cell-free cytosolic homogenate was prepared as previously described (15). CK activity is expressed as phosphocholine associated cpm synthesized per minute per mg of homogenate protein (cpm/min/mg).

RESULTS

Effects of GM–CSF and SLF on choline-lipid labelling. Factor starved-M07e cells were stimulated for various times with GM–CSF, SLF, or GM–CSF plus SLF, pulse-labelled with [^{3}H]-choline and the lipids extracted. Figure 1A shows the effects of these cytokines on [^{3}H]-choline accumulation into choline-lipids in M07e cells. Both cytokines induced an increase in labelling of PC, lysoPC (LPC), and SPH with the combination of GM–CSF plus SLF being the most effective (except in SPH labelling). SPH labelling stimulated by the combination of GM–CSF plus SLF for six hours was no more effective than SLF alone. A statistically significant effect of the combination of GM–CSF plus SLF on PC labelling could be observed after ten minutes of stimulation (plus one hour labelling), while effects on LPC and SPH required longer stimulation times before statistical significance was observed.

Ten minutes after stimulation with the growth factors, there was a slight decrease in CDP-choline labelling and a concomitant slight increase in choline pool labelling after SLF stimulation, and no significant change in phosphocholine labelling (Fig. 1B). After one hour of stimulation with GM–CSF or GM–CSF plus SLF, the CDP-choline labelling level was lower than control (unstimulated), and choline pool labelling fell significantly when stimulated with SLF or SLF plus GM–CSF. After six hours of stimulation with GM–CSF plus SLF, choline pool labelling continued to be lower while phosphocholine and CDP-choline labelling was increased by stimulation with GM–CSF or SLF alone and was increased additively when stimulated with GM–CSF plus SLF. There was a considerable increase in the rate of [^{3}H]-choline accumulation into choline lipids and intermediates between 1 and 6 h (Fig. 1). In general, SLF was a more potent inducer of choline lipid metabolism than GM–CSF, but simultaneous stimulation with both cytokines was the most potent inducer.

The effects of 24 h of growth-factor stimulation were also examined (Fig. 2). However, the 24-h experiments were treated separately. We have previously demonstrated that the viability of growth-factor starved M07e cells did not change during the previous 6 h stimulation time irrespective of the stimulant (13). But after 24 h, the viability of the medium control cultures fell to approximately 60%. The viable cell density was adjusted to 1×10^6 cells/mL for all experiments, but because of this difference, the 24-h effects could not be directly compared to the 6-h (or earlier) data, and are thus shown separately. After 24 h of stimulation, SLF was equipotent to GM–CSF in inducing choline-lipid labelling. There was a true synergistic response to stimulation with the combination of SLF plus GM–CSF in PC, LPC, and phosphocholine labelling, but not in choline and CDP-choline labelling, which had no significant difference from control when stimulated with the single cytokine, but was increased slightly by stimulation with the combination of GM–CSF plus SLF. SPH labelling was increased additively with the combination of GM–CSF plus SLF.

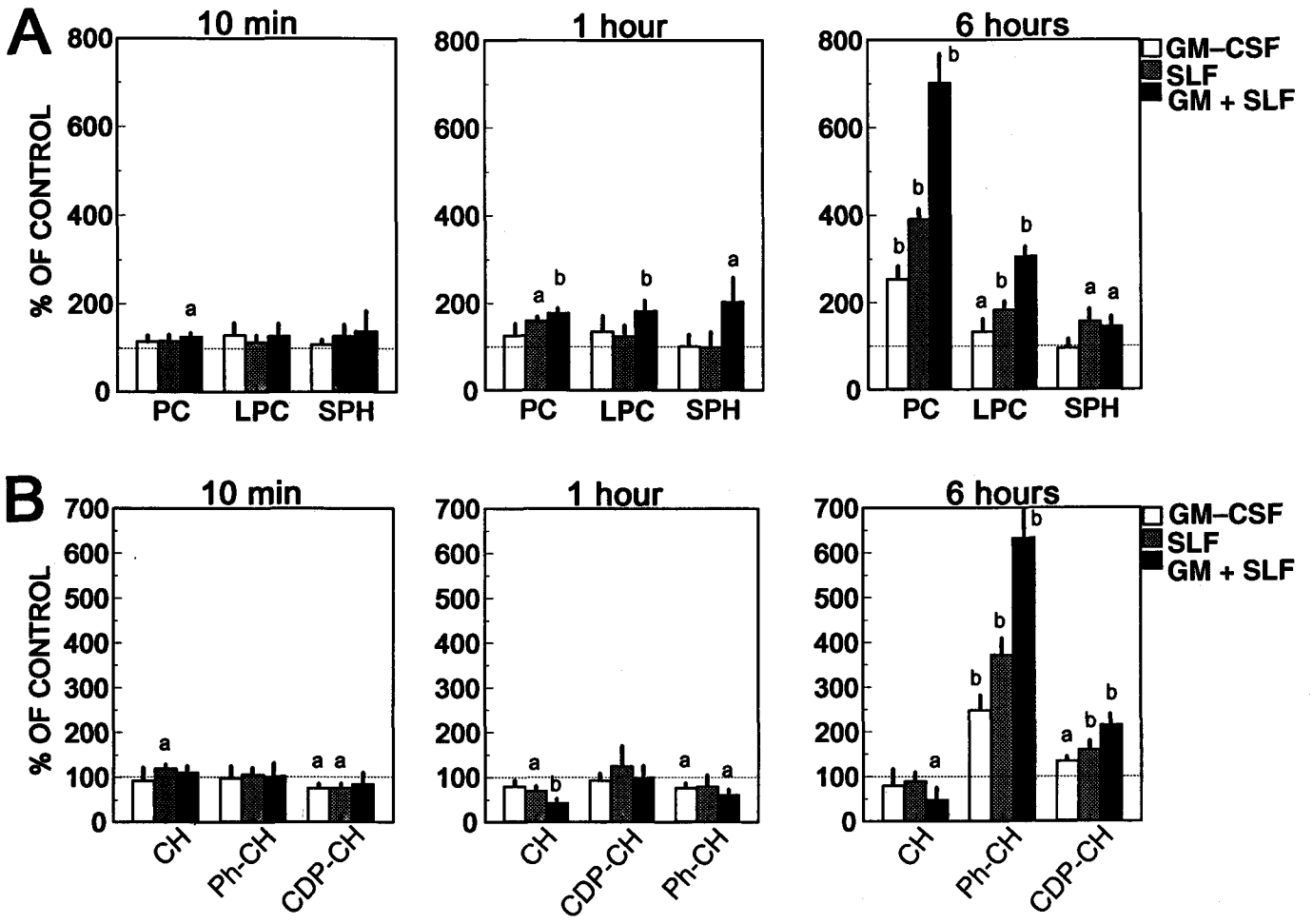


FIG. 1. Effect of cytokines on [^{3}H]-choline accumulation into M07e cellular choline–lipids and precursors. Factor starved-M07e cells were stimulated with granulocyte-macrophage colony-stimulating factor (GM–CSF), steel factor (SLF), or GM–CSF plus SLF for the indicated times, and cellular choline–lipids and aqueous intermediates were labelled with [^{3}H]-choline according to the Methods and Materials section. The cells were then harvested by centrifugation and the lipids extracted and separated by thin-layer chromatography. Spots corresponding to the indicated choline–lipid (A) or intermediates (B) were scraped and counted. Results are expressed as percent of control cpm (± SEM) of radioactivity recovered per spot per 1×10^7 cells. Statistical analysis compared to control: a = $P < 0.05$; b = $P < 0.001$; n = 3 experiments with triplicate determinations. Control cpm for phosphatidylcholine (PC), lysoPC (LPC), and sphingomyelin (SPH) was 2055, 107, and 55, respectively. Control cpm for choline (CH), phosphocholine (Ph-CH), and CDP-choline (CDP-CH) was 603, 6341, and 132, respectively.

Effects of GM–CSF and SLF on PL labelling by FAs. The pattern of FA labelling of different PL classes in unstimulated M07e cells is shown in Table 1. PC, PI, phosphatidic acid (PA), and phosphatidylethanolamine accumulated the most [^{3}H]-AA, followed by LPC, phosphatidylserine (PS), diacylglycerol (DAG), and SPH. PC also accumulated the most [^{3}H]-MA, followed by phosphatidylethanolamine and PA. SPH, LPC, PI, PS, and DAG accumulated the least MA.

The effects of growth-factor stimulation of FA labelling are shown in Table 2. GM–CSF and SLF had similar effects in inducing increased accumulation of [^{3}H]-FAs into different PL classes. However, this effect was enhanced in cells that were simultaneously stimulated with GM–CSF plus SLF. In some cases there was an additive or greater-than-additive effect when cells were stimulated with GM–CSF plus SLF. All PL classes studied showed growth-factor induced increases in [^{3}H]-MA accumulation with SLF being generally more potent in this effect than GM–CSF. PS labelling showed the greatest response (~12-fold increase), and PC showed the least (~ 2-fold). In contrast, only PA and DAG consistently had significantly enhanced accumulation of [^{3}H]-AA, which was induced only when SLF was present. Also, there was no further enhancement of this effect when stimulation was done with GM–CSF plus SLF.

With respect to the AA/MA ratio, only a slight change in relative magnitude between the different PL classes occurred after growth factor stimulation, but in general, GM–CSF and/or SLF reduced the AA/MA ratio in all PL classes studied, with PS displaying the greatest effect (8% of unstimulated control).

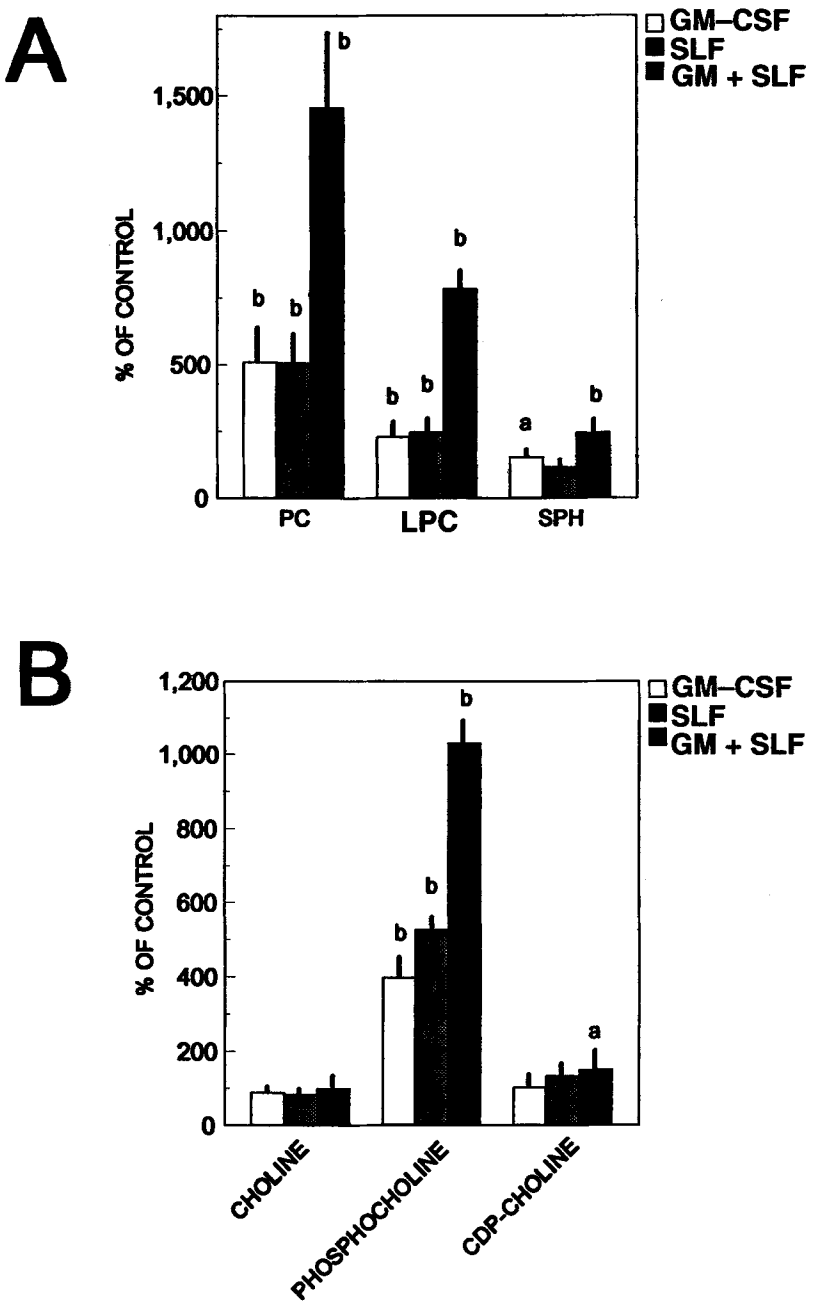


FIG. 2. Effect of cytokine stimulation for 24 h on [^{3}H]-choline accumulation into M07e cellular choline-lipids and precursors. Factor starved-M07e cells were treated, labelled, harvested, and analyzed exactly as in Figure 1, except that cells were stimulated with cytokines for 24 h. Statistical analysis and significance were the same as Figure 1. Control cpm for PC, LPC, and SPH (A) was 505, 51, and 41, respectively and for choline, phosphocholine, and CDP-choline (B) was 335, 1372, and 72, respectively. Abbreviations as in Figure 1.

Effects of GM–CSF and SLF on cytosolic CK activity. We previously proposed that GM–CSF and SLF upregulate CK activity in M07e cells (13). This was based on the observation that phosphocholine labelling increased and choline pool labelling decreased after GM–CSF and/or SLF treatment for six hours. Therefore we examined the effects of GM–CSF and/or SLF stimulation on cytosolic CK activity (Fig. 3). CK activity increased in a time-dependent fashion, with SLF being more effective than GM–CSF in this effect. Ten minutes after stimulation with GM–CSF plus SLF, there was a significant decrease in CK activity compared to control, but this increased to 251 ± 38% of control after six hours of stimulation with GM–CSF plus SLF. Although SLF alone was more potent in this effect than GM–CSF alone, the combination of GM–CSF plus SLF was no more potent than SLF alone, indicating that stimulated CK activity was not synergistically increased by the combination of GM–CSF plus SLF.

TABLE 1
Unstimulated [3H]-Fatty Acid Labelling of Selected Factor Starved-M07e Cellular Lipids[a]

	CPM/Spot × 1000	
	Arachidonic acid	Myristic acid
Phosphatidylcholine	239 ± 62	84.0 ± 9.3
Sphingomyelin	0.82 ± 0.10	2.3 ± 0.3
Lysophosphatidylcholine	17.6 ± 1.6	2.7 ± 0.16
Phosphatidylethanolamine	169 ± 19	15.5 ± 1.9
Phosphatidylserine	2.6 ± 0.7	1 ± 0.28
Phosphatidic acid	183 ± 13	8.9 ± 0.44
Phosphatidylinositol	194 ± 23	1.4 ± 0.11
Diacylglycerol	1 ± 0.05	0.5 ± 0.1

[a]Factor starved-M07e cells were labelled with either [^{3}H]-arachidonic acid or [^{3}H]-myristic acid according to the Methods and Materials section. Cellular lipids were extracted, separated by two-dimensional thin-layer chromatography, and spots corresponding to the indicated lipids were scraped and counted. Results are expressed as cpm/spot (from 1 × 10^7 cells) ± SEM from two experiments each done in triplicate.

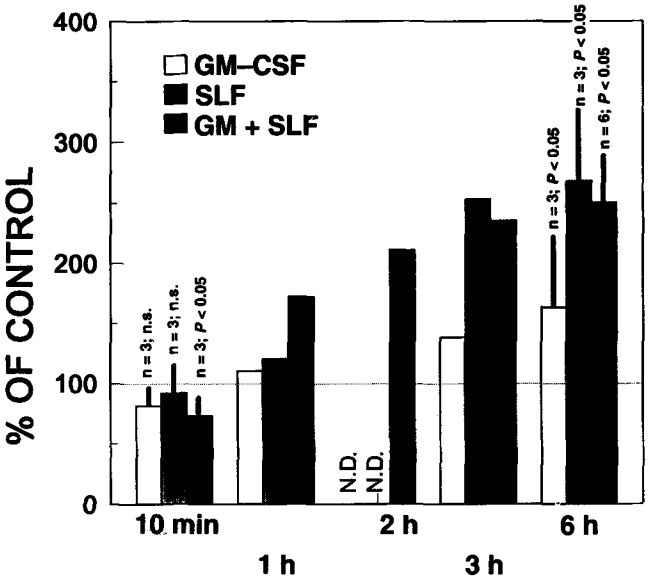


FIG. 3. Effect of cytokines on factor starved-M07e cytosolic choline kinase activity. Factor starved-M07e cells were stimulated for the indicated times, the cells harvested, cytosol prepared, and choline kinase assayed according to the Methods and Materials section. Results were expressed as percent of control activity. The number of experiments (with triplicate determinations; n, the statistical significance (*P*), and SEM bars are shown where appropriate. Other results are the means of a single experiment with triplicate determinations. N.D. = not done. Unstimulated control values for 10 min, 1, 2, 3, and 6 h were 8397 ± 660, 1432, 1539, 1257, and 1416 ± 142 cpm/min/mg cytosolic protein, respectively.

DISCUSSION

It is clear from this study and from our previous study (13) that GM–CSF and SLF upregulate choline lipid metabolism in M07e cells. Simultaneous stimulation with saturating concentrations of both GM–CSF and SLF results in a true synergistic stimulation of [^{3}H]-choline flux through the PC biosynthetic and degradative pathways (i.e., turnover). Because both of these cytokines are known to use second messenger molecules which can be derived from PC (i.e., DAG) (9,17) as part of their signalling mechanism, this induced synergistic PC turnover may have relevance to synergistic signalling mechanisms, and this suggests that a PC hydrolytic cycle (18) may be part of the synergistic proliferative response of M07e cells to these two cytokines. Even though some significant effects could be seen after 10 min of stimulation time, a large increase in the effects of GM–CSF and/or SLF on PC metabolism occurred between one and six hours, suggesting that these effects may be considered "delayed" response elements (19). Support for this notion comes from the delayed (one hour) increase in CK activity. Further experiments are required to determine the mechanism of upregulation of CK activity (i.e., transcriptional regulation-vs.-post-transcriptional modification regulation). It is also noteworthy that the increase in CK activity stimulated by the combination of GM–CSF plus SLF was no more than SLF alone (i.e., not synergistic or even additive). Yet at six hours of stimulation, the increased phosphocholine labelling was at least additive,

TABLE 2
Effect of Cytokines on Induced [^{3}H]-Fatty Acid Labelling of Selected Factor Starved-M07e Cellular Lipids[a]

	Fold change compared to unstimulated control					
	GM–CSF		SLF		GM + SLF	
	Arachidonic acid	Myristic acid	Arachidonic acid	Myristic acid	Arachidonic acid	Myristic acid
Phosphatidylcholine	n.s./n.s.	2.5×/1.6×	n.s./n.s.	1.4×/1.8×	n.s./n.s.	2.0×/2.0×
Sphingomyelin	n.s./1.4×	3.3×/2.6×	n.s./n.s.	2.1×/3.7×	n.s./n.s.	3.5×/3.8×
Lysophosphatidylcholine	n.s./n.s.	1.9×/1.7×	n.s./n.s.	1.9×/1.9×	n.s./n.s.	2.6×/2.1×
Phosphatidylethanolamine	n.s./n.s.	2.2×/5.0×	1.7×/n.s.	3.2×/6.0×	n.s./n.s.	5.5×/7.2×
Phosphatidylserine	0.5×/n.s.	2.6×/6.1×	n.s./n.s.	5.3×/10.4×	n.s./n.s.	11.2×/13.3×
Phosphatidic acid	n.s./1.4×	1.6×/4.3×	1.2×/1.8×	3.1×/6.0×	1.6×/1.8×	5.7×/8.2×
Phosphatidylinositol	1.3×/n.s.	2.2×/4.9×	n.s./n.s.	4.0×/6.5×	n.s./n.s.	8.6×/7.2×
Diacylglycerol	1.3×/n.s.	2.2×/1.5×	2.0×/1.7×	2.2×/2.0×	2.3×/1.4×	3.3×/3.8×

[a]Factor starved-M07e cells were stimulated with cytokines as described in the Methods and Materials section and the cells labelled, extracted, separated, and analyzed as in Table 1. Results are expressed as fold increase over control cpm/spot (Table 1). The results from two experiments are shown separated by a slash (/). For clarity, only those results that were statistically significant ($P < 0.05$) are shown. Other results (n.s.) were not statistically significant ($P > 0.05$). SEMs are not shown for clarity, but did not exceed the percentages of those shown for the control (Table 1). GM–CSF, granulocyte-macrophage colony-stimulating factor; SLF, steel factor.

suggesting that the proportion of label in the phosphocholine pool which was contributed by the hydrolytic component of the synergistic turnover rate (i.e., PLC) at six hours may be more significant than the contribution by the biosynthetic component. It therefore seems unlikely that CK upregulation is solely responsible for the observed synergistic increase in phosphocholine labelling.

Saturating amounts of SLF were, in general, a more potent inducer of FA labelling of various PL classes than saturating amounts of GM–CSF. This is interesting because SLF is a much weaker single mitogen than GM–CSF in M07e cells (7,14). Therefore the upregulation of PC metabolism by SLF is not just simply due to upregulation of the general metabolic state of the cell in lieu of mitosis. However, it is interesting that after 24 h of stimulation, GM–CSF and SLF were equipotent in induction of PC and LPC labelling. PC metabolism has been demonstrated to be closely correlated to cell-cycle events (4). As SLF has been recently suggested to be a "competence factor" (20) or a "survival factor" (21), whereas GM–CSF appears to be a more complete mitogen [competence and progression (20)], it is possible that the difference in relative potency of GM–CSF and SLF at different times after stimulation may reflect the differences of these cytokines in regulating the cell-cycle status of M07e cells, resulting in different ratios of biosynthesis and degradation of PC. Even after 24 h of stimulation, SLF was still more effective in stimulating phosphocholine labelling. This is noteworthy because phosphocholine has been suggested as a potential second messenger-like molecule which appears to be related to other competence factors, such as platelet-derived growth factor (22).

The level of GM–CSF-induced labelling of PC with [^{3}H]-choline was approximately the same as the induced level of [^{3}H]-MA labelling. SLF and SLF plus GM–CSF (synergistic) induced labelling with [^{3}H]-choline (~7-fold increase) could not be accounted for by either [^{3}H]-AA or [^{3}H]-MA labelling (~2-fold increase). Therefore, other species of PC also must be involved in GM–CSF and/or SLF-induced synergistic upregulation of PC metabolism. Alternatively, the mechanism of introduction of FAs and choline into PC may be independently regulated, and there may be differential activation of these pathways. SLF, but not GM–CSF, also was able to stimulate the incorporation of AA into only two PLs, PA and DAG. This may significantly contribute to the differential effects of these two cytokines, especially as phospholipase D has already been implicated in SLF signal transduction. Phospholipase D catalyzes the degradation of PC to PA, and PA is a precursor to DAG.

GM–CSF and SLF both induced a asymmetric patterns of FA labelling of various PL classes (i.e., AA vs. MA labelling). This differential activation of metabolism of different PL species may have relevance to the generation of different species of lipid second messenger molecules (i.e., DAGs). Different lipid second messengers have already been suggested as differentially activating different isoforms of PKC (23). Further work is required to determine which species of PC are involved in GM–CSF and/or SLF stimulated PC turnover and the relevance of this to the generation of different species of second messenger molecules which could contribute to the diversity of effects of different cytokines.

Both GM–CSF- and SLF induced asymmetric FA labelling of PS; GM–CSF plus SLF induced at least an additive or cooperative effect on PS labelling by [^{3}H]-MA. Of all the PL classes studied, PS labelling was most affected by these cytokines, with an approximately 12-fold increase in [^{3}H]-MA labelling. The AA/MA ratio was also reduced to only 8% of the unstimulated control. As PS is usually found to be one of the least abundant components of eukaryotic cell membranes and because it displays extreme leaflet asymmetry (24), this effect of GM–CSF plus SLF may be of significance in the signalling process. This is especially interesting in view of the finding of the potential requirement of PS in the membrane binding, translocation, and activation of protein kinase-C (PKC) (25). Therefore, the cooperative effect of GM–CSF plus SLF on upregulation of PS metabolism also may have relevance to signal transduction mechanisms involving PKC as well as other inducible enzyme activities [such as Raf-1 kinase (26)] that are translocated to various membrane surfaces *via* PS. Further work is required to determine which species are involved in cytokine-induced PS metabolism and whether this phenomenon plays a role in induced membrane translocation and cytokine activation of PKC, Raf-1, or other signalling molecules.

ACKNOWLEDGMENTS

We thank Shantay Glover and Becki Miller for typing the manuscript. This work was supported by U.S. Public Health Service Grants R37 CA36464, R01 HL46549, and R01 HL49202 from the National Cancer Institute and the National Institutes of Health to H.E.B.

REFERENCES

1. Berridge, M.J., and Irvine, R.F. (1989) Inositol Phosphates and Cell Signalling, *Nature 341*, 197–205.
2. Billah, M.M., and Anthes, J.C. (1990) The Regulation and Cellular Functions of Phosphatidylcholine Hydrolysis, *Biochem. J. 269*, 281–291.
3. Kolesnick, R., and Golde, D.W. (1994) The Sphingomyelin Pathway in Tumor Necrosis Factor and Interleukin-1 Signaling, *Cell 77*, 325–328.
4. Jackowski, S. (1994) Coordination of Membrane Phospholipid Synthesis with the Cell Cycle, *J. Biol. Chem. 269*, 3858–3867.
5. Broxmeyer, H.E., and Williams, D.E. (1988) The Production of Myeloid Blood Cells and Their Regulation During Health and Disease, *CRC Crit. Rev. Oncol./Hematol. 8*, 173–226.
6. Broxmeyer, H.E. (1992) Update: Biomolecule-Cell Interactions and the Regulation of Myelopoiesis, in *Concise Rev. Clin. and Exp. Hematol* (Murphy, M.J., ed.), pp. 119–149, Alpha Med Press, Dayton.
7. Miyazawa, K., Hendrie, P.C., Mantel, C., Wood, K., Ashman, L.K., and Broxmeyer, H.E. (1991) Comparative Analysis of Signaling Pathways Between Mast Cell Growth Factor (c-kit ligand) and Granulocyte-Macrophage Colony-Stimulating Factor in a Human Factor-Dependent Myeloid Cell Line Involves

Phosphorylation of Raf-1, GTPase-Activating Protein and Mitogen-Activated Protein Kinase, *Exp. Hematol. 19*, 1110–1123.
8. Hallek, M., Druker, B., Lepisto, E.M., Wood, K., Ernst, T.J., and Griffin, J.D. (1992) Granulocyte-Macrophage Colony-Stimulating Factor and Steel Factor Induce Phosphorylation of Both Unique and Overlapping Signal Transduction Intermediates in a Human Factor-Dependent Hematopoietic Cell Line, *J. Cell. Physiol. 153*, 176–186.
9. Nishimura, M., Kaku, K., Azuno, Y., Okafugi, K., Inoue, Y., and Kaneko, T. (1992) Stimulation of Phosphoinositol Turnover and Protein Kinase C Activation by Granulocyte-Macrophage Colony-Stimulating Factor in HL-60 Cells, *Blood 80*, 1045–1051.
10. Horie, M., and Broxmeyer, H.E. (1993) Involvement of Immediate-Early Gene Expression in the Synergistic Effects of Steel Factor in Combination with Granulocyte-Macrophage Colony-Stimulating Factor or Interleukin-3 on Proliferation of a Human Factor-Dependent Cell Line, *J. Biol. Chem. 268*, 968–973.
11. Rottapel, R., Reedijk, M., Williams, D.E., Lyman, S.D., Anderson, D.K., Pawson, T., and Bernstein, A. (1991) The Steel/W Transduction Pathway: Kit Autophosphorylation and Its Association with a Unique Subset of Cytoplasmic Signaling Proteins Is Induced by the Steel Factor, *Mol. Cell. Biol. 11*, 3043–3051.
12. Broxmeyer, H.E., Maze, R., Miyazawa, K., Carow, C., Hendrie, P.C., Cooper, S., Hangoc, G., Vadhan-Raj, S., and Lu, L. (1991) The Kit Receptor and Its Ligand, Steel Factor, as Regulators of Hemopoiesis, *Cancer Cells 3*, 480–487.
13. Mantel, C., Luo, Z., Hendrie, P., and Broxmeyer, H.E. (1993) Steel Factor and Granulocyte-Macrophage Colony Stimulating Factor Act Together to Enhance Choline-Lipid Turnover During Synergistically Stimulated Proliferation of the Human Factor Dependent Cell Line, M07e, *Biochem. Biophys. Res. Comm. 197*, 978–984.
14. Hendrie, P.C., Miyazawa, K., Yang, Y.C., Langefeld, C.D., and Broxmeyer, H.E. (1991) Mast Cell Growth Factor (c-kit ligand) Enhances Cytokine Stimulation of Proliferation of the Human Factor-Dependent Cell Line, M07e, *Exp. Hematol. 19*, 1031–1037.
15. Mantel, C.R., Shultz, A.R., Miyazawa, K., and Broxmeyer, H.E. (1993) Kinetic Selectivity of Cholinephosphotransferase in Mouse Liver: the K_m for CDP-Choline Depends on Diacylglycerol Structure, *Biochem. J. 289*, 815–820.
16. Warden, C.H., and Friedkin, M. (1985) Regulation of Choline Kinase Activity and Phosphatidylcholine Biosynthesis by Mitogenic Growth Factors in 3T3 Fibroblasts, *J. Biol. Chem. 260*, 6006–6011.
17. Koike, T., Hirai, K., Morita, Y., and Nozawa, Y. (1993) Stem Cell Factor-Induced Signal Transduction in Rat Mast Cells. Activation of Phospholipase D But Not Phosphoinositide-Specific Phospholipase C in c-kit Receptor Stimulation, *J. Immunol. 151*, 359–366.
18. Exton, J. (1990) Signaling Through Phosphatidylcholine Breakdown, *J. Biol. Chem. 265*, 1–4.
19. Murray, A., and Hunt, T. (1993) *The Cell Cycle: An Introduction*, pp. 111–116, W.H. Freeman and Co., New York.
20. Hallek, M., Ando, K., Eder, M., Slattery, K., Ajchenbaum-Cymbalista, F., and Griffin, J.D. (1994) Signal Transduction of Steel Factor and Granulocyte-Macrophage Colony-Stimulating Factor: Differential Regulation of Transcription Factor and G1 Cyclin Gene Expression, and of Proliferation in the Human Factor-Dependent Cell Line M07, *Leukemia 8*, 740–748.
21. Caceres-Cortes, J., Rajotte, D., Dumouchel, J., Haddad, P., and Hoang, T. (1994) Product of the Steel Locus Suppresses Apoptosis in Hemopoietic Cells. Comparison with Pathways Activated by Granulocyte Macrophage Colony-Stimulating Factor, *J. Biol. Chem. 269*, 12084–12091.
22. Cuadrado, A., Carnero, A., Dolfi, F., Jimenez, B., and Lacal, J.C. (1993) Phosphorylcholine: A Novel Second Messenger Essential for Mitogenic Activity of Growth Factors, *Oncogene 8*, 2959–2968.
23. Liscovitch, M., and Cantley, L.C. (1994) Lipid Second Messengers, *Cell 77*, 329–334.
24. Zachowski, A. (1993) Phospholipids in Animal Eukaryotic Membranes: Transverse Asymmetry and Movement, *Biochem. J. 294*, 1–14.
25. Quest, A.F., and Bell, R.M. (1994) The Regulatory Region of Protein Kinase C Gamma. Studies of Phorbol Ester Binding to Individual and Combined Functional Segments Expressed as Glutathione S-Transferase Fusion Proteins Indicate a Complex Mechanism of Regulation by Phospholipids, Phorbol Esters, and Divalent Cations, *J. Biol. Chem. 289*, 20000–20012.
26. Ghosh, S., Xie, W.Q., Quest, A.F.G., Mabrouk, G.M., Strum, J.C., and Bell, R.M. (1994) The Cysteine-Rich Region of raf-1 Kinase Contains Zinc, Translocates to Liposomes, and Is Adjacent to a Segment That Binds GTP-ras, *J. Biol. Chem. 269*, 10000–10007.

[Received February 1, 1995, and in revised form May 11, 1995; Revision accepted May 22, 1995]

Plasma Fatty Acids and Lipids in Two Separate, But Genetically Comparable, Icelandic Populations

Gudrún V. Skúladóttir[a,*], Sigrún Gudmundsdóttir[a], Gunnlaugur B. Ólafsson[a], Stefán B. Sigurdsson[a], Nikulás Sigfússon[b] and Jóhann Axelsson[a]

[a]Department of Physiology, University of Iceland, 101 Reykjavík, Iceland and [b]The Heart Preventive Clinic of the Icelandic Heart Association, Reykjavík, Iceland

ABSTRACT: Levels of serum lipids and lipoproteins, and the fatty acid composition of plasma phospholipids, were measured in two genetically comparable, but widely separated, populations. The 1975 mortality rates for ischemic heart disease were significantly higher in one of these populations, the Manitoban residents of pure Icelandic descent, than in the other, a rural population from Northeastern Iceland. Two study populations, Icelanders and Icelandic–Canadians, were drawn from these larger populations. The study populations were matched for age and sex and divided into three age groups, 20–39, 40–59, and 60–69 years. In comparison to the Icelandic–Canadians, the Icelanders exhibited significantly higher levels of total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol, but lower triglyceride levels. Their plasma phospholipids contained significantly lower levels of saturated fatty acids (SFA), monounsaturated fatty acids, and n-6 polyunsaturated fatty acids (PUFA); but their n-3 PUFA levels were three times as high. It was additionally found that fatty acid composition of plasma phospholipids differed among Icelanders of different ages. SFA levels were significantly lower, and n-6 PUFA levels significantly higher, in the 20–39 year group than in the 60–69 year group, possibly due to different dietary fat consumption patterns between generations. No corresponding age-related difference in the fatty acid composition of plasma phospholipids was found in the Icelandic–Canadian study population. As the Icelandic and Icelandic–Canadian groups are assumed to be genetically similar, the biochemical differences between them are evidently due to environmental, probably dietary, differences. The findings indicate that n-3 PUFA may be cardioprotective in the context of an otherwise atherogenic diet. *Lipids 30,* 649–655 (1995).

This investigation is part of a larger, and on-going, comparative study, begun in 1979, of cardiovascular risk factors in two genetically comparable, but geographically and culturally separate populations, one Icelandic and the other Icelandic–Canadian (1,2).

*To whom correspondence should be addressed at Department of Physiology, University of Iceland, Vatnsmýrarveg; 16, IS-107 Reykjavík, Iceland.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; HDL, high-density lipoproteins; LDL, low-density lipoproteins; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

By the late 19th century, Icelanders had lived in virtual isolation from the rest of the world for 1,000 years, and the population of Iceland was thus unusually homogeneous. In the period between 1870 and 1914, some 18,000 Icelanders, about 20% of the national population in that period, migrated to North America. Because of meticulous record-keeping, the names, professions, and ages of these emigrants, the farms from which they came, the ships they traveled with, and the places in North America where they settled are known (3,4). Many of these Icelanders migrated from Fljótsdalshérad, a rural district in Northeastern Iceland, to the Interlake District, just north of Winnipeg, Manitoba; and hundreds of individuals currently living there are pure descendants of the Icelandic immigrants from Fljótsdalshérad. A large number of these purely-descended Icelandic–Canadians have been identified through genealogical records (5,6) and questionnaires. Individuals living in Fljótsdalshérad were invited to participate in the study if their families had been living in Fljótsdalshérad for at least three generations (1). The environmental homogeneity within the sample was increased by selecting those who live in the same district as their parents and grandparents did. There is every reason to suppose that these two study populations thus identified are highly comparable genetically. This presumed genetic comparability has been confirmed by anthropomorphic studies (1,7). The existence of these two genetically comparable populations living in wide separation from one another and in quite dissimilar surroundings provides an exceptional opportunity for comparative studies aimed at distinguishing environmental from hereditary influences, for example on the prevalence of ischemic heart disease.

In 1975, crude mortality rates for ischemic heart disease were significantly higher in the Icelandic–Canadian communities of Manitoba than in Iceland, for both females and males (1,8). Because the explanation for this difference is evidently not genetic, environmental factors—dietary factors in particular—are indicated. Indeed, the traditional Icelandic diet differs considerably from the normal diet of Icelandic–Canadians. As an effect of diet, it is the lipid profile which is thought to have the most to do with the risk of cardiovascular disease (CVD). The present study investigates serum levels of total choles-

Copyright © 1995 by AOCS Press

terol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and plasma phospholipid fatty acids of two genetically similar study populations; these profiles exhibit significant differences. We attempt to link the study parameters with CVD risk.

MATERIALS AND METHODS

Subjects. Two study populations were constructed, one consisting of 119 subjects (60 females and 59 males) drawn from the rural district of Fljótsdalshérad, in Northeastern Iceland, and a second consisting of 119 subjects (59 females and 60 males) drawn from a previously-identified pool of residents of the Interlake District of Manitoba who are pure descendants of immigrants from Fljótsdalshérad (1,2). The Icelandic study population was constructed first; five-year cohorts of persons aged 20–69 years, each cohort containing approximately six females and six males, were selected. The Icelandic–Canadian study population was then constructed by selecting matching cohorts. The two resulting study populations were thus well matched with respect to age and sex, down to the level of the five-year cohorts. For the purpose of analysis in the present study, the two populations were divided into three age groups—20.0–39.9, 40.0–59.9, and 60.0–69.9 years (Table 1).

Overnight fasting blood samples were collected by venipuncture into Vacutainer® tubes (Sarstedt, Nünbrecht, Germany) containing EDTA for fatty acid determinations and no anticoagulant for determinations of lipids and lipoproteins. Plasma or serum was prepared immediately, frozen, and flown in cold packs to the Heart Preventive Clinic (Reykjavík, Iceland), where it was stored at –22°C until analyzed.

Analysis of lipids and lipoproteins. Total serum cholesterol was determined on a Cobas Mira (Roche Diagnostic Systems, Hoffmann-la Roche Ltd., Basel, Switzerland) automatic analyzer by an enzymatic colorimetric method as described by Allain *et al.* (9) and Trinder (10). Serum triglyceride was determined on the Cobas Mira by an enzymatic colorimetric method as described by Fossati and Prencipe (11) and McGowan *et al.* (12). Serum HDL cholesterol was determined on the Cobas Mira according to the method of Burstein *et al.* (13) and Lopes-Virella *et al.* (14). The concentration of LDL cholesterol was calculated as follows:

$$[\text{LDL cholesterol}] = [\text{total cholesterol}] - ([\text{HDL cholesterol}] + [\text{triglyceride}] \times 0.37) \quad [1]$$

Commercial control sera were used for internal quality control, and external quality control was performed by the World Health Organization Lipid Reference Centre in Prague.

Analysis of phospholipid fatty acids. Plasma lipids were extracted with chloroform/methanol (2:1, vol/vol) by the method of Sperry and Brand (15) as modified by Nelson (16). The antioxidant butylated hydroxytoluene was added to the extraction medium at 5 mg/100 mL. Phospholipids were separated on thin-layer plates (Adsorbosil H; Alltech, Deerfield, IL) and developed in a solvent system consisting of petroleum ether/diethylether/acetic acid (80:20:1, by vol). The phospholipid fatty acids were methylated by the method of Morrison and Smith (17) using 14% boron trichloride/methanol (Sigma

TABLE 1
Body Weight, Body Mass Index (BMI), Triglycerides, Total Cholesterol, High-Density Lipoprotein (HDL) Cholesterol, and Low-Density Lipoprotein (LDL) Cholesterol in Icelanders and in Icelandic–Canadians[a]

	Icelanders			Icelandic–Canadians					
	20.0–39.9 (30.7)[c] n = 42 (F = 21, M = 21)	40.0–59.9 (50.8) n = 55 (F = 27, M = 28)	60.0–69.9 (65.1) n = 22 (F = 12, M = 10)	20.0–39.9 (30.8) n = 43 (F = 21, M = 22)	40.0–59.9 (50.5) n = 55 (F = 27, M = 28)	60.0–69.9 (64.8) n = 21 (F = 11, M = 10)	*P* value[b] Group	Age	G × A
Body weight (kg)	74.9 ± 2.6	77.2 ± 2.3	80.0 ± 3.6	75.4 ± 2.4	81.4 ± 2.1	81.6 ± 3.2	NS	NS	NS
BMI (kg/m^2)	25.3 ± 0.7	26.7 ± 0.6	28.5 ± 0.9	25.6 ± 0.7	27.5 ± 0.5	28.8 ± 1.3[f]	NS	<0.0005	NS
Triglycerides (mM)	1.09 ± 0.14	1.33 ± 0.13	1.13 ± 0.09	1.29 ± 0.08	1.70 ± 0.12	1.46 ± 0.11	0.007	0.014	NS
Total cholesterol (mM)	5.4 ± 0.10	6.2 ± 0.10[e]	6.9 ± 0.20[d]	5.0 ± 0.20	5.4 ± 0.10	5.9 ± 0.20	<0.0005	<0.0005	NS
HDL cholesterol (mM)	1.3 ± 0.04	1.3 ± 0.05	1.4 ± 0.08	1.04 ± 0.03	0.9 ± 0.03	1.01 ± 0.06	<0.0005	NS	NS
LDL cholesterol (mM)	3.7 ± 0.10	4.5 ± 0.10[e]	5.1 ± 0.20[d]	3.4 ± 0.10	3.8 ± 0.10	4.3 ± 0.20	<0.0005	<0.0005	NS

[a]Values are mean ± SEM of n determinations, where n represents the number individuals in each age group. Sex distribution among the age groups is shown in parentheses (F = female, M = male).
[b]Significance calculated by two-way analysis of variance, NS = not significant; G = group of all Icelanders (n = 119) or group of all Icelandic–Canadians (n = 119), A = age.
[c]Represents age period in years, and mean age is given in parentheses.
[d]$P < 0.02$, compared to the youngest and middle age groups, Scheffe post-hoc test.
[e]$P < 0.01$; [f]$P < 0.02$, compared to the youngest age groups, Scheffe post-hoc test.

Chemical Co., St. Louis, MO). The methyl esters were analyzed using a gas chromatograph (Hewlett-Packard 5890A; Hewlett-Packard, Palo Alto, CA) equipped with a capillary column, 25 m × 0.2 mm i.d., coated with a 0.25 μm film of 5% diphenyl- and 95% dimethyl polysiloxane. The gas chromatograph was programmed to have an initial oven temperature of 160°C rising in three increments to 235°C. The carrier gas was hydrogen at a flow rate of 1 mL/min. The fatty acid methyl ester peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Applied Science Division, Milton Roy Company, State College, PA).

All measurements in the comparative studies of Icelanders and Icelandic–Canadians have been carried out by the same personnel, using the same techniques and equipment, and the chemical analyses have been performed in the same laboratory.

Statistical analysis. The influence of environment (Fljótsdalshérad vs. Interlake District) and age on serum lipids, lipoproteins, and fatty acid composition of plasma phospholipids in all subjects studied was examined by two-way analysis of variance using SYSTAT (18) general linear model procedures. To determine the significant difference in level of lipids, lipoproteins, and phospholipid fatty acids between the age groups within each population, one-way analysis of variance was used, followed by application of Scheffe post-hoc test. Pearson's correlation coefficient was used to measure the correlation between age and the percentage of fatty acids in plasma phospholipids. Differences were considered significant at $P < 0.05$.

RESULTS

The study parameters for persons of different ages in the two populations are shown in the tables and the figure. There were no significant differences in body weight and body mass index between the two study populations (Table 1). The body mass index of the oldest age group of Icelandic–Canadians was significantly higher than that of the youngest age group. The Icelandic study population exhibited significantly lower triglyceride levels and significantly higher levels of total cholesterol, LDL cholesterol, and HDL cholesterol than the Icelandic–Canadian study population (Table 1).

Age-related differences between total cholesterol and LDL cholesterol levels were found among the Icelanders. Within this population the oldest age group exhibited significantly higher total cholesterol and LDL cholesterol levels than the youngest and middle groups. The middle group contained significantly higher levels of total cholesterol and LDL cholesterol than the youngest group. No significant age-related differences in lipid levels were found among the Icelandic–Canadians.

Sex-related differences between lipid levels were found in both study populations (data not shown). Triglyceride levels were significantly higher in males than in females; 1.41 ± 0.15% vs. 1.00 ± 0.06% ($P = 0.046$), in the Icelanders, and 1.74 ± 0.11% vs. 1.26 ± 0.06% ($P = 0.009$), in the Icelandic–Canadians. Within the Icelandic study population, total cholesterol levels were significantly lower in males than in females, 5.9 ± 0.1% vs. 6.3 ± 0.2% ($P = 0.039$). HDL cholesterol levels were also significantly lower in males than in females; 1.17 ± 0.04% vs. 1.43 ± 0.04% ($P < 0.0005$) in Icelanders, and 0.90 ± 0.03% vs. 1.04 ± 0.04% ($P = 0.004$) in Icelandic–Canadians.

The two study populations differed markedly in the fatty acid composition of plasma phospholipids (Table 2). Seven out of the eleven fatty acids analyzed differed significantly between the two populations. The levels of 20:5n-3 and 22:6n-3 were three times higher in Icelanders than in Icelandic–Canadians, in all age groups (2.20 ± 0.09 and 4.30 ± 0.11% vs. 0.72 ± 0.03 and 1.38 ± 0.06%, respectively). There were no significant differences in the n-6 polyunsaturated fatty acids (PUFAs), 20:3n-6 and 20:4n-6, and in the saturated fatty acids, 14:0 and 18:0. The interaction between group and age was significant in six fatty acids (Table 2).

Age-related differences between fatty acid levels in plasma phospholipids were found within both study populations. In the Icelandic study population, the level of 14:0 was significantly lower, and the levels of 18:2n-6 and 20:4n-6 were significantly higher in the youngest age group than in the middle and oldest age groups. The plasma phospholipids of the youngest age group of Icelandic–Canadians contained significantly lower levels of 20:5n-3 than the middle and oldest age groups.

In both study populations, significant sex-related differences between fatty acid levels in plasma phospholipids were found only in saturated fatty acids (SFAs) (data not shown). SFA levels were significantly higher in females than in males, 46.6 ± 0.37% vs. 43.3 ± 0.47% ($P = 0.002$) in Icelanders and 49.0 ± 0.33% vs. 46.9 ± 0.36% ($P < 0.005$) in Icelandic–Canadians.

Figure 1 shows the relationship between age and levels of SFA (A), n-6 PUFA (B) and n-3 PUFA (C) in plasma phospholipids of Icelanders and Icelandic–Canadians of both sexes. In Icelanders, SFA levels were positively correlated ($r = 0.528$, $P < 0.0005$), and n-6 PUFA levels correlated negatively ($r = -0.573$, $P < 0.0005$) with age. A small positive correlation between n-3 PUFA levels and age in Icelanders was observed ($r = 0.273$, NS). No relationship was found between age and levels of SFA ($r = 0.042$, NS), n-6 PUFA ($r = -0.051$, NS), and n-3 PUFA ($r = 0.231$, NS) in the plasma phospholipids of Icelandic–Canadians.

DISCUSSION

Mortality from coronary heart disease is relatively low among Icelanders, even though they exhibit a lipid profile which is normally considered atherogenic. It is recognized that many chronic forms of CVD have long development periods, and total serum cholesterol level has been thought to play a central role in development of atherosclerosis. Hypercholesterolemia in childhood is one of the factors that may affect the development of CHD with aging (19,20). Icelandic

TABLE 2
Plasma Phospholipid Fatty Acid Composition in Icelanders and Icelandic–Canadians[a]

	Icelanders			Icelandic–Canadians					
	20.0–39.9 (30.7)[b] n = 42	40.0–59.9 (50.8) n = 55	60.0–69.9 (65.1) n = 22	20.0–39.9 (30.8) n = 43	40.0–59.9 (50.5) n = 55	60.0–69.9 (64.8) n = 21	*P* value[c] Group	Age	G × A
Fatty acids									
14:0	0.57 ± 0.02[d]	0.71 ± 0.03	0.70 ± 0.03	0.54 ± 0.02	0.58 ± 0.03	0.55 ± 0.03	NS	0.012	0.005
16:0	28.67 ± 0.38	30.40 ± 0.35	32.23 ± 0.38	32.67 ± 0.35	31.51 ± 0.30	32.50 ± 0.58	<0.0005	0.001	<0.0005
16:1n-7	0.56 ± 0.04	0.53 ± 0.03	0.51 ± 0.07	0.86 ± 0.04	0.92 ± 0.04	0.97 ± 0.06	0.021	NS	NS
18:0	13.71 ± 0.20	14.14 ± 0.19	15.29 ± 0.20	14.78 ± 0.29	15.40 ± 0.18	15.90 ± 0.38	NS	<0.0005	NS
18:1n-9	11.77 ± 0.24	12.00 ± 0.23	12.66 ± 0.36	14.15 ± 0.27	14.01 ± 0.26	14.24 ± 0.46	0.001	NS	NS
18:2n-6	21.34 ± 0.51[e]	19.11 ± 0.48	17.34 ± 0.70	18.97 ± 0.36	19.22 ± 0.35	18.48 ± 0.48	<0.0005	<0.0005	<0.0005
20:3n-6	2.04 ± 0.09	1.82 ± 0.06	1.63 ± 0.10	2.01 ± 0.09	2.36 ± 0.08	2.13 ± 0.11	NS	NS	0.001
20:4n-6	6.14 ± 0.16[e]	5.45 ± 0.14	5.00 ± 0.18	6.83 ± 0.30	6.92 ± 0.21	6.46 ± 0.22	NS	0.001	NS
20:5n-3	1.82 ± 0.11	2.31 ± 0.13	2.72 ± 0.25	0.55 ± 0.03	0.75 ± 0.04[g]	0.99 ± 0.13[f]	0.009	<0.0005	0.043
22:5n-3	1.01 ± 0.04	1.17 ± 0.03	1.21 ± 0.07	0.51 ± 0.03	0.63 ± 0.03	0.53 ± 0.03	0.005	0.001	0.024
22:6n-3	4.04 ± 0.11	4.41 ± 0.18	4.48 ± 0.32	1.29 ± 0.10	1.43 ± 0.07	1.46 ± 0.14	<0.0005	0.047	NS
Total									
SFA	42.95 ± 0.45	45.24 ± 0.49	48.21 ± 0.43	47.99 ± 0.45	47.49 ± 0.38	48.95 ± 0.58	<0.0005	<0.0005	<0.0005
MUFA	12.34 ± 0.24	12.53 ± 0.24	13.17 ± 0.40	15.01 ± 0.29	14.93 ± 0.27	15.21 ± 0.51	0.001	NS	NS
n-6 PUFA	29.52 ± 0.43	26.38 ± 0.48	23.97 ± 0.63	27.81 ± 0.46	28.50 ± 0.38	27.06 ± 0.65	<0.0005	<0.0005	<0.0005
n-3 PUFA	6.87 ± 0.22	7.88 ± 0.31	8.41 ± 0.60	2.36 ± 0.14	2.81 ± 0.12	2.98 ± 0.25	<0.0005	<0.0005	NS
n-6/n-3	4.48 ± 0.16	3.66 ± 0.18	3.21 ± 0.26	13.28 ± 0.69	11.02 ± 0.47	10.27 ± 0.78	<0.0005	<0.0005	NS

[a]Data are expressed as relative weight percent of total fatty acids. Each value represents the mean ± SEM of n determinations, where n represents the number of individuals in each age group. Sex distribution among the age groups is shown in Table 1. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]Represents age period in years and mean age is given in parentheses.
[c]Significance calculated by two-way analysis of variance, NS = not significant, G = group of all Icelanders (n = 119) or group of all Icelandic–Canadians (n = 119), A = age.
[d]$P < 0.05$; [e]$P < 0.01$, compared to the middle and oldest age groups, Scheffe post-hoc test.
[f]$P < 0.02$, compared to the youngest and middle age groups, Scheffe post-hoc test.
[g]$P < 0.05$, compared to the youngest age groups, Scheffe post-hoc test.

youngsters, ages 6–19, exhibit one of the highest levels of total serum cholesterol yet reported, 5.31 ± 0.86 mM (mean ± SD) (4). However, mortality from CVD is relatively low among Icelanders, which indicates that mean total cholesterol levels fail to reflect cardiovascular risk in a population and are a poor predictor of individual risk (4).

In the face of this conundrum, it is worth noting that Icelanders hold the European record in fish consumption (73 g/day/person), and the use of cod liver oil as a daily food supplement is widespread (21). Consumption of fish and fish oil rich in the n-3 PUFA, 20:5n-3, and 22:6n-3, has been shown to have a beneficial effect on plasma lipid profiles, thereby reducing the risk of CVD (22–27). The effects of fish oil consumption on the levels of total serum cholesterol and LDL cholesterol are less clear (28,29). Some studies have indicated that consumption of fish containing moderate amounts of n-3 PUFA may cause a deleterious rise in LDL cholesterol levels in normotriglyceridemic males, whereas other studies have found no change (30,31). The relatively high levels of LDL cholesterol exhibited by the Icelandic study population lend support to the argument that the consumption of fish and fish oil increases LDL cholesterol (Table 1).

Low levels of serum triglycerides in humans have been observed to correlate with consumption of fish and fish oil (29). The high Icelandic consumption of these foods may thus explain why serum triglyceride levels were significantly lower in the Icelandic study population than in the Icelandic–Canadian population (Table 1).

It has been maintained that relatively high plasma phospholipid levels of 20:5n-3 and 22:6n-3 are strictly related to diet (32–34). The relatively high levels of plasma phospholipid n-3 PUFA observed in the Icelandic study population may thus be taken to reflect the dietary intake of these two n-3 PUFA found in fish and fish oil, whereas the 30% lower levels of n-3 PUFA observed in the Icelandic–Canadians evidently reflect low dietary levels of n-3 PUFA in the ordinary Canadian diet (Table 2).

Mean levels of plasma phospholipid 20:4n-6, the precursor of the thrombogenic and vasospastic thromboxane A_2, have been shown to be twice as high in Canadians and in Americans (32,33) as in Nordic populations (35–37). It has been suggested that relatively low levels of plasma phospholipid 20:4n-6 may be genetically controlled and independent of diet (33). In the present study, no significant difference was observed in plasma phospholipid 20:4n-6 levels between the two genetically similar study populations, which is consistent with this suggestion (Table 2).

It is generally supposed that the prevalence of CVD correlates positively with the proportion of dietary energy supplied by saturated fatty acids and negatively with the proportion of

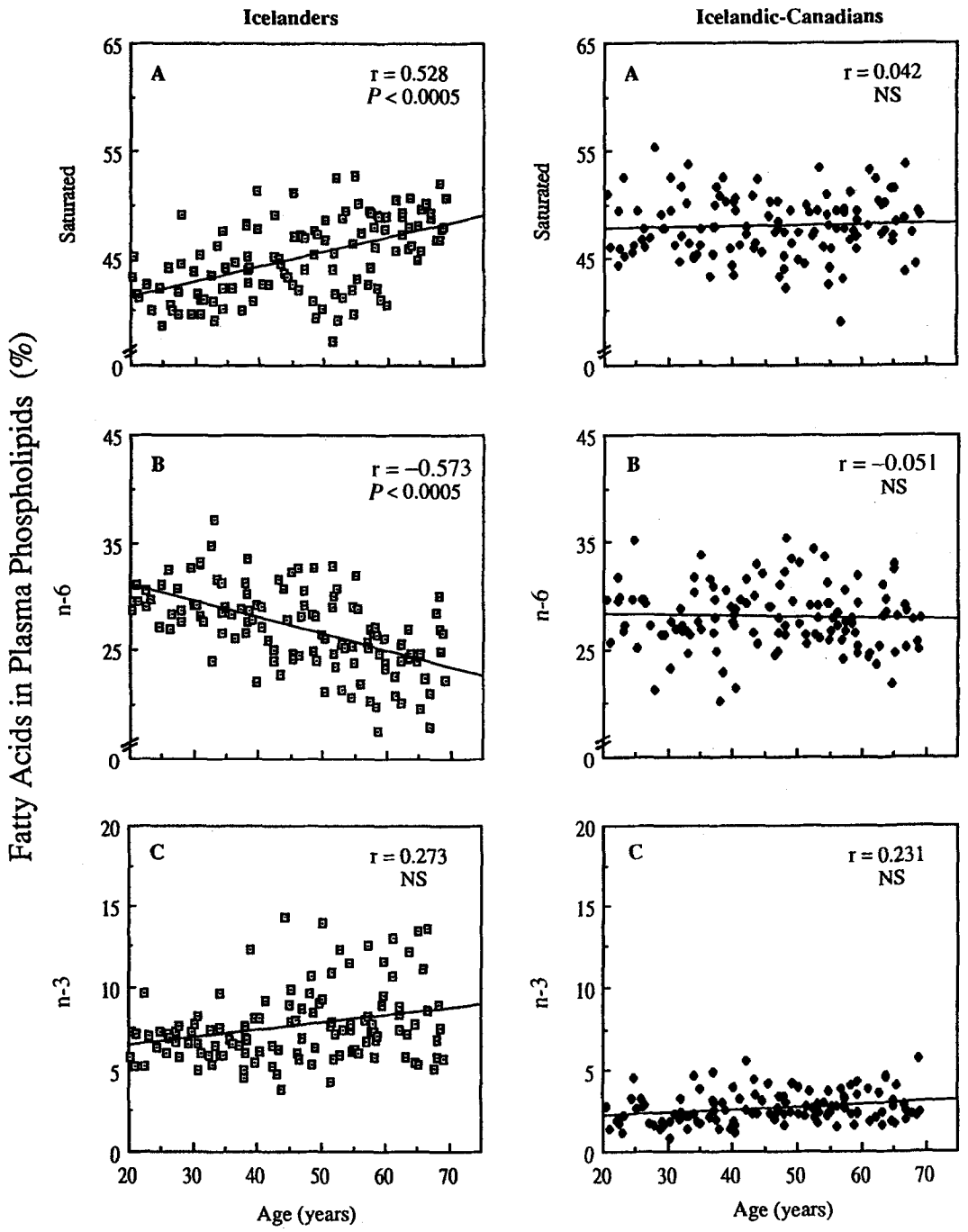


FIG. 1. Relationship between age and percentage of saturated fatty acids (A), n-6 polyunsaturated fatty acids (PUFAs) (B), and n-3 PUFA (C) of plasma phospholipids in Icelanders (left) and Icelandic–Canadians (right). Each point represents an individual subject. Saturated, sum % (14:0 + 16:0 + 18:0); n-6 PUFA, sum % (18:2 + 20:3 + 20:4) n-6; n-3 PUFA, sum % (20:5 + 22:5 + 22:6) n-3. NS, not significant.

dietary n-6 PUFA (38). In Iceland, fat contributes 41% of total dietary energy, with the contribution of saturated fat amounting to 20% (21). However, the contribution of total dietary fat energy in Canada is 38% and saturated fat is estimated to 13% (39). Until the late 1970s, the traditional Icelandic diet consisted of local products, i.e., fish, lamb, and dairy products rich in cholesterol and saturated fat (40). Grains, fruits, and vegetables, which were generally imported and expensive, contributed less to the Icelandic diet. In the Icelandic study population, plasma phospholipid SFA levels were found to increase with age, while in the Icelandic–Canadian population, no age-related difference was observed (Table 2 and Fig. 1). Canadian–Icelanders of all ages exhibited SFA levels of plasma phospholipids similar to those observed in Icelanders 60 years of age and older. Since the late 1970s, a public health campaign in Iceland has advocated the reduction of

fatty meats and dairy fats in the diet and the increased consumption of vegetable oils and grains. The lower levels of SFA and n-3 PUFA, and higher levels of n-6 PUFA observed in plasma phospholipids of younger Icelanders (Table 2 and Fig. 1) probably reflect the effects of this campaign (4). Older persons in Fljótsdalshérad who tend to eat the traditional Icelandic high-fat diet and consume cod liver oil exhibit higher levels of SFA and n-3 PUFA and lower levels of n-6 PUFA in plasma phospholipids than do younger Icelanders (Table 2 and Fig. 1). The study indicates that the dietary habits of Icelandic–Canadians are rather stable, as no age-related differences in SFA, n-6 PUFA, or n-3 PUFA of plasma phospholipids were observed (Table 2 and Fig. 1). That such age-related differences in plasma phospholipid fatty acids in the Icelanders are due to changes in dietary patterns rather than effects of aging as such is supported by the work of Holman *et al.* (32).

Of interest in this connection is that lamb, which is a major element in the traditional Icelandic high-fat diet, contains more n-3 PUFA than has been generally realized. Due to short and often hard summers in Iceland, it is customary to supplement the ewes' diet with fish meal, herring, or fish oil during breeding season and prior to the lambing season in the spring. The maternal dietary n-3 PUFA are incorporated into the lean muscle phospholipids of the lamb meat, which in lambs from Fljótsdalshérad contains 7% of n-3 PUFA (unpublished data from our laboratory).

Serum triglyceride level, now recognized as an independent risk factor for CVD (41), is increasing rapidly in Icelanders (42). A 10-year follow-up study (1980–1990) on a subgroup of the population in Fljótsdalshérad (ages 20–60 years) revealed that the cardiovascular risk attributable to triglyceride levels and atherogenic indices have increased markedly (43), evidently reflecting changes in dietary fat intake.

It is generally agreed that n-3 PUFA play an essential role in human growth and development throughout life and are a necessary part of the diet (44). They may be instrumental in preventing the development of CVD (45). The present study supports the idea that n-3 PUFA, such as Icelanders obtain through their consumption of fish, fish oil, and Icelandic lamb, can afford protection from atherosclerotic disease, even in the face of a diet that in other respects may be counted as atherogenic (46).

ACKNOWLEDGMENTS

This study was supported by grants from the University of Iceland Research Foundation and the Icelandic Council of Science. Thanks to Mikael M. Karlsson for valuable help with the manuscript.

REFERENCES

1. Axelsson, J., Pálsson, J.Ó.P., Pétursdóttir, G., Sigfússon, N., and Way, A.B. (1981) in *Circumpolar Health 81* (Harvald, B., and Hart Hansen, J.P., eds.) Report Series 33, pp. 201 -205, Copenhagen.
2. Axelsson, J., Sigfússon, N., Way, A.B., and Pétursdóttir, G. (1981) in *Circumpolar Health 81* (Harvald, B., and Hart Hansen, J.P., eds.) Report Series 33, pp. 304–307, Copenhagen.
3. Kristinsson, J.H. (1983) *Vesturfaraskrá 1870–1914 (A Record of Immigrants from Iceland to America 1870–1914),* Reykjavík, Institute of History, University of Iceland.
4. Axelsson, J., Karlsson, M., Pétursdóttir, G., Asgeirsdottir, A.G., Olafsson, O., Sigfusson, N., Way, A.B., Sigvaldason, H., and Sigurdsson, S.B. (1990) in *Circumpolar Health 90* (Postl, B.D., Gilbert, P., Goodwill, J., Moffatt, M.E.K., O'Neil, J.D., Sarsfield, P.A., and Young, T.K., eds.) pp. 509–511, Canada.
5. Gerrard, N.S. (1985) *Icelandic River Saga,* Saga Publications, Arborg, Manitoba.
6. Gislason, L., Johannson, G., Sigurdson, S., and Sigvaldason, A. (1987) *A Century Unfolds,* The Arborg Historical Society, Arborg, Manitoba.
7. Pálsson, J.Ó.P. (1988) in *Yearly Report of the National Research Council of Iceland 1986–87* (Ragnarsdóttir, G.A., ed.) pp. 62–63, Visindarád, Reykjairk, Iceland.
8. World Health Organization World Health Statistics Annual 1977, (1977) *I. Vital Statistics and Causes of Death,* Geneva.
9. Allain, C.C., Poon, L.S., Chon, C.S.G., Richmond, U., and Fu, P.C. (1974) *Clin. Chem. 20,* 470–475.
10. Trinder, P. (1969) *Ann. Clin. Biochem. 6,* 24–27.
11. Fossati, P., and Prencipe, L. (1982) *Clin. Chem. 28,* 2077–2080.
12. McGowan, M.W., Artiss, J.D., Strandbergh, D.R., and Zak, B. (1983) *Clin. Chem. 29,* 538–542.
13. Burstein, M., Scholnick, H.R., and Morfin, R. (1970) *J. Lipid Res. 11,* 583.
14. Lopes-Virella, M.F., Stone, P., Ellis, S., and Colwell, J.A. (1977) *Clin. Chem. 23,* 882–884.
15. Sperry, W.M., and Brand, F.C. (1955) *J. Biol. Chem. 213,* 69–76.
16. Nelson, G.J. (1979) in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism* (Nelson, G.J., ed.), pp. 10–12, Krieger, Huntington.
17. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600–608.
18. SYSTAT: Statistics, Version 5.2 Edition, SYSTAT, Inc., 1992, Evanston.
19. Connor, W.E., and Connor, S.L. (1990) *Adv. Intern. Med. 35,* 139–172.
20. Åkerblom, H.K., Viikari, J., and Kouvalainen, K. (1985) *Acta Pædiatr. Scand. Suppl. 318,* 5–6.
21. Steingrímsdóttir, L. (1993) *Scand. J. Nutr. 37,* 10–12.
22. Dyerberg, J., and Bang, H.O. (1979) *The Lancet ii,* 433–435.
23. Bang, H.O., and Dyerberg, J. (1980) in *Advances in Nutritional Research* (Draper, H.H., ed.), pp. 1–23, Plenum Press, New York.
24. Dyerberg, J. (1981) *Phil. Trans. R. Soc. Lond. B 294,* 373–381.
25. Kinsella, J.E. (1987) *Am. J. Cardiol. 60,* 23G–32G.
26. Kromhout, D., Bosschieter, E.B., and Coulander, C. de L. (1985) *N. Engl. J. Med. 312,* 1205–1209.
27. Norell, S.E., Ahlbom, A., Feychting, M., and Pedersen, N.L. (1986) *Br. Med. J. 293,* 426.
28. Herold, P.M., and Kinsella, J.E. (1986) *Am. J. Clin. Nutr. 43,* 566–598.
29. Harris, W.S. (1989) *J. Lipid Res. 30,* 785–807.
30. Gerhard, G.T., Patton, B.D., Lindquist, S.A., and Wander, R.C. (1991) *Am. J. Clin.. Nutr. 54,* 334–339.
31. Lindgren, F.T., Adamson, G.L., Shore, V.G., Nelson, G.J., and Schmidt, P.C. (1991) *Lipids 26,* 97–101.
32. Holman, R.T., Smythe, L., and Johnson, S. (1979) *Am. J. Clin. Nutr. 32,* 2390–2399.
33. Bates, C., van Dam, C., Horrobin, D.F., Morse, N., Huang, Y-S., and Manku, M.S. (1985) *Prost. Leuk. Med. 17,* 77–84.

34. Bjerve, K.S., Brubakk, A.M., Fougner, K.J., Johnsen, H., Midthjell, K., and Vik, T. (1993) *Am. J. Clin. Nutr. 57 (suppl.)*, 801S–806S.
35. Dyerberg, J., Bang, H.O., and Hjörne, N. (1975) *Am. J. Clin. Nutr. 28,* 958–966.
36. Kirkeby, K., Ingvaldsen, P., and Bjerkedal, I. (1972) *Acta Med. Scand.* 192, 513–519.
37. Skuladottir, G., Hardarson, Th., Sigfusson, N., Oddsson, G., and Gudbjarnason, S. (1985) *Acta Med. Scand.* 218, 55–58.
38. Jacobsen, B.K., and Thelle, D.S. (1988) *Am. J. Epidemiol.* 127, 923–932.
39. Health and Welfare Canada (1990) *Action Towards Healthy Eating: The Report of the Communications/Implementation Committee,* Minister of Supply and Services Canada.
40. Sigfusson, N., Sigvaldason, H., Steingrimsdottir, L., Gudmundsdottir, I.I., Stefansdottir, I., Thorsteinsson, Th., and Sigurdsson, G. (1991) *Br. Med. J. 302,* 1371–1375.
41. Castelli, W.P., (1986) *Am. Heart J.* 112, 432–437.
42. Ólafsson, Ó., Sigfússon, N., Davidsson, D., and Björnsson, O.J. (1988) *Arc. Med. Res., Suppl. 1, Vol. 47,* 466–468.
43. Axelsson, J., Gudmundsdóttir, S., Sigurdsson, S.B., Pétursdóttir, G., Way, A.B., and Ólafsson, Ó. (1994) *Arc. Med. Res., Supp. 2, Vol. 53,* 590–591.
44. Simopoulos, A.P. (1991) *Am. J. Clin. Nutr. 54,* 438–463.
45. Lands, W.E.M. (1991) in *Health Effects of ω3 Polyunsaturated Fatty Acids in Seafoods* (Simopoulos, A.P., Kifer, R.R., Martin, R.E., Barlow, S.M., eds.) Vol. 66, pp. 177–194, World Rev. Nutr. Diet, Basel, Karger.
46. Leaf, A. (1993) *Omega-3 News Vol. VIII,* 1–4.

[Received November 30, 1994, and in revised form March 20, 1995; Revision accepted April 10, 1995]

Dietary Fatty Acids, Membrane Transport, and Oxidative Sensitivity in Human Erythrocytes

David E. Mills[a,b,*], Mahadev Murthy[c] and William R. Galey[b]

[a]Scientific Laboratory Division, New Mexico Department of Health, Albuquerque, New Mexico 87196-4700, [b]Department of Physiology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131 and [c]Department of Foods and Nutriton, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada

ABSTRACT: This study examined effects of dietary n-3 fatty acids on age-related changes in erythrocyte anion transport and susceptibility to oxidation. Blood was drawn from healthy adult volunteers before and after six weeks' supplementation (nine/group) with 4.0 g/day of safflower oil (containing 2.9 g n-6 fatty acids) or fish oil (containing 1.2 g long-chain n-3 fatty acids). Following density separation of young and old erythrocytes, membrane anion transport and cell membrane lipid composition were measured. Oxidative damage was measured in erythrocyte ghosts exposed to a free radical generator. Fish oil significantly increased 16:0 and 20:5n-3 in ghosts of both young and old cells, and 22:5n-3 and 22:6n-3 in old cells alone. Safflower oil increased 16:0, 18:0, 18:1n-9, and 22:5n-6 in ghosts of young cells only. The age-dependent increase in membrane anion transport ($P < 0.01$) was decreased by dietary fish oil supplementation, but not by safflower oil supplementation. Safflower oil and fish oil increased the susceptibility of both young and old erythrocytes to oxidative damage by free radical generation ($P < 0.001$).

Lipids 30, 657–663 (1995).

The aging of the circulating erythrocyte is associated with numerous structural and functional changes, including altered membrane fatty acid composition (1–6), solute transport (7), antigen expression (8,9), decreased deformability, and increased fragility (10,11). These changes may interfere with the ability of the senescent erythrocyte to deliver oxygen to peripheral tissues, or to contribute to its removal from the circulation (7,8).

It is well established that the types of polyunsaturated fatty acids consumed in the diet can influence erythrocyte membrane phospholipid composition and function in humans (12–20). Studies examining the effects of dietary supplementation with 20 and 22 carbon n-3 fatty acids, such as those found in fish oils (20:5n-3 and 22:6n-3), have generally observed an increase in membrane n-3/n-6 ratio, a decrease in whole blood viscosity, and an increase in membrane deformability in human erythrocytes (2–10). Furthermore, we recently reported that dietary supplementation with fish oil reversed the age-related decrease in the *in vitro* filterability of old circulating erythrocytes in humans, restoring it to the level of young cells (21). These changes were accompanied by an increased incorporation of long-chain n-3 fatty acids into the membrane lipids of both young and old erythrocytes.

While these data suggest a possible role for dietary n-3 fatty acids in reversing age-related changes in erythrocyte function, more information concerning their effect on age-related membrane function, e.g., transport properties, would be useful. In addition, it would be of interest to determine whether the increased levels of long-chain, highly unsaturated fatty acids in the erythrocyte membrane make it more susceptible to damage from oxidative stressors.

The purpose of the present study was to investigate the effects of dietary supplementation with long-chain n-3 fatty acids (fish oil), as compared with n-6 fatty acids [safflower oil (SAF)], on erythrocyte membrane anion transport, and susceptibility to oxidative damage of young and old erythrocytes, separated by density centrifugation, in humans.

MATERIALS AND METHODS

Subjects. Healthy adult volunteers were recruited from the University of Waterloo (Ontario, Canada) student population. After the elimination of smokers, individuals taking medication or oral contraceptives, and those currently under medical treatment, 18 subjects (9 males and 9 females, 21–41 yr, mean 27 yr) were enrolled in the study.

Protocol. All participants were randomized within gender into one of two treatment groups in which their normal diet was supplemented with 4.0 g/day of either SAF, high in 18:2n-6 (4 males and 5 females) or fish oil, high in 20:5n-3 and 22:6n-3 (5 males and 4 females), in the form of 0.5-mL gelatin capsules, for a six-week period (unpublished pilot studies in our laboratory have shown that membrane phospholipid fatty acid changes occur within this time frame). Both fatty acid supplements, whose compositions are shown in Table 1, also contained 26 mg α-tocopherol acetate/g oil (30 IU/g) to protect against oxidation. The intervention was

*To whom correspondence should be addressed at Scientific Laboratory Division, Department of Health, P.O. Box 4700, Albuquerque, NM 87196-4700.

Abbreviations: DBI, double-bond index; MDA, malondialdehyde; RBC, red blood cells; SAF, safflower oil; TBARS, thiobarbituric acid-reactive substances.

Copyright © 1995 by AOCS Press

TABLE 1
Fatty Acid Composition (% total fatty acids) of Safflower Oil and Fish Oil Used in the Study (Ref. 21)

Fatty acid	Safflower oil	Fish oil
14:0	—	7.6
16:0	6.6	1.4
16:1n-7	0.1	9.0
18:0	2.3	3.0
18:1n-9	11.1	12.6
18:2n-6	72.3	3.7
18:3n-3	0.4	—
20:4n-6	—	0.6
20:5n-3	—	18.6
22:5n-3	—	2.4
22:6n-3	—	12.7
n-6	72.3	4.3
n-3	0.4	33.7

conducted under double-blind conditions. Prior to and immediately following the dietary supplementation period, a 60-mL blood sample was drawn into heparinized tubes from an antecubital vein, after an overnight fast, for analysis of lipid composition, anion transport, and sensitivity to oxidative damage of the erythrocyte membranes of young and old circulating erythrocytes. In addition, both prior to and following the dietary supplementation period, all subjects completed a food frequency survey (the dietary portion of the Health Habits and History Questionnaire, National Cancer Institute, National Institutes of Health, Bethesda, MD) in order to (i) evaluate the dietary fat intake of the subjects in the two treatment groups and to (ii) determine any significant shifts in dietary fat consumption that might have occurred during the dietary supplementation period.

Preparation and age separation of erythrocytes. After removing the plasma and buffy coat, packed erythrocytes were washed twice with 0.05 M phosphate-buffered saline (pH 7.4). Cells were separated by age according to the method of Murphy (22). In this procedure, 12 mL aliquots of packed cells, adjusted to a hematocrit of 85–90%, were centrifuged for 60 min at 27,000 × *g* in a fixed angle rotor at 27°C. After discarding the supernatant, the top (least dense) and bottom (most dense) 2.1 mL (~20%) of erythrocytes, i.e., the youngest and the oldest, were aspirated from each tube. Of these fractions, 0.1 mL were drawn off for measurement of erythrocyte number (23) and wet and dry weight in order to calculate erythrocyte water content. The remaining 2.0 mL of each erythrocyte fraction were taken for preparation of erythrocyte ghosts, according to the method of Steck (24) and used for measurements of fatty acid composition and oxidative sensitivity.

Measurement of anion transport. Membrane anion transport was determined in young and old erythrocytes according to the method of Zanner and Galey (7). Briefly, 1.0 mL of erythrocytes were diluted to a hematocrit of 25% and incubated with 3 mL buffer (80 mEq/l NaCl, 5 mEq/l KCl, 10 mEq/l Na_2SO_4, 5 mEq/l Na-Hepes, 5 mM glucose, and 100 mM sucrose, adjusted to pH 7.4) containing 10 μCi/mol $^{35}SO_4$ at 37°C for 2.5 h. The incubate was then centrifuged at 6,000 × *g* for 10 min, the buffer removed, and the packed cells washed twice. The labeled, packed erythrocytes were reconstituted to 5% hematocrit in buffer and incubated in a shaking water bath at 37°C for 2 h. At 10-min intervals, samples of 1 mL were drawn, centrifuged (Eppendorf microfuge; Eppendorf, Madison, WI) for 15 s, and 0.1 mL of the supernatant was added to 5.0 mL scintillation cocktail and counted in a liquid scintillation counter (LS1801; Beckman Instruments, Fullerton, CA). The rate coefficient of $^{35}SO_4$ efflux (SO_4^{2-}-Cl^- exchange), k, was calculated by linear regression analysis of the plot of $\ln(1-a_t/a_\infty)$ vs. time, where a_t and a_∞ indicate the extracellular radioactivity at sampling time t, and equilibrium, respectively. The negative of the slope of this plot is equal to the rate coefficient of the SO_4 efflux (7).

Measurement of oxidative sensitivity. A superoxide generating system was developed by adding 1 mL of erythrocyte ghost suspension, containing 5 mg protein, to 200 μmol xanthine (Sigma, St. Louis, MO) and 0.2 U xanthine oxidase (Sigma) in a total reaction volume of 1.5 mL. This mixture was incubated at 37°C for 24 h, at which time 1.5 mL trichloroacetic acid (15%) was added. The mixture was then centrifuged at 1,500 × *g* for 10 min and the supernatant removed. One mL thiobarbituric acid (0.67%) was added to the supernatant, which was heated for 25 min in a boiling water bath to develop the color. The production of thiobarbituric acid-reactive substances (TBARS), primarily malondialdehyde (MDA), was measured using a spectrophotometer (Shimadzu, Tokyo, Japan) at 532 nm. TBARS were determined from a standard curve prepared from a TBA–MDA conjugate of known concentration.

Lipid analysis of erythrocyte ghosts. Fatty acid composition of the total ghost lipid fraction was determined by gas–liquid chromatography. Lipids were extracted from 0.4 mL ghost suspension with chloroform/methanol, as previously described (25). Following the addition of an internal standard (10 μg 17:0; Nu-Chek-Prep, Elysian Fields, MN), samples were transmethylated with methanolic sulfuric acid (6%) at 90°C for 2 h. Fatty acid methyl esters were then analyzed on a Perkin Elmer (Norwalk, CT) gas chromatograph (model 8420) equipped with a flame-ionization detector and a 30 m capillary column (Supelcowax 10; Supelco Ltd., Oakville, Ontario, Canada). A temperature ramped program was used, as previously described (25). Phospholipid content of ghost membranes was estimated from inorganic phosphorus content (P_i). Thirty μL ghost suspension were digested with 1.2 mL perchloric acid over 4 h at 90°C, and then 1.5 mL ammonium molybdate (2.5%) and 0.2 mL Fiske-Subbarow reagent were added and the mixture heated in a boiling water bath for 7 min. The resulting color was read at 830 nm and compared with a standard curve generated using KH_2PO_4. The P_i was then multiplied by 25 to estimate the phospholipid content of the ghosts.

Statistics. Data comparing dietary intake patterns were analyzed using a two-way analysis of variance (treatment × time) on repeated measures. Data comparing membrane lipid

and phospholipid composition, cell water content, membrane anion exchange, and TBARS production were analyzed using a three-way analysis of variance (treatment × time × cell age) on repeated measures. Where a significance of $P < 0.05$ was achieved, specific comparisons were then made using Tukey's highly significant differences test.

RESULTS

One male subject from the fish oil intervention group did not complete the protocol for reasons unrelated to the study, leaving a sample size of 8 (4 males and 4 females) in the fish oil group. The dietary fat intake of both experimental groups prior to and following dietary intervention (not including the supplements) is shown in Table 2. All values represent mean ± SEM. Neither treatment nor time significantly affected the estimated daily intake of total, saturated, or monounsaturated fat, 18:2n-6 or dietary polyunsaturated fat/saturated fat ratio.

Red cell membrane fatty acid composition. The total membrane fatty acid compositions of density separated (young and old) erythrocyte ghosts prior to and following six weeks' dietary supplementation with either SAF or fish oil are shown in Table 3. There were no age-related differences in the fatty acid composition of ghosts from young vs. old cells, with the exception of an increase in 14:0 ($P < 0.05$) in the ghosts from old cells of the SAF group. Dietary supplementation with SAF increased the content of 16:0, 18:0, 18:1n-9, and 22:5n-6 ($P < 0.05$) of ghosts from young cells and decreased the content of 14:0 ($P < 0.05$) from ghosts of old cells. It also tended to increase the n-6 fatty acids, although this did not attain statistical significance ($P < 0.07$). Supplementation with fish oil increased the content of 16:0 ($P < 0.05$) and 20:5n-3 ($P < 0.01$) of ghosts from young cells. It also increased the content of 16:0, 20:5n-3, 22:5n-3 ($P < 0.01$), and 22:6n-3 ($P < 0.05$) and decreased the content of 18:3n-3 ($P < 0.05$) from the ghosts from old cells. Consistent with this was an increase in the n-3 fatty acids in fish oil-supplemented groups ($P < 0.05$). The double-bond index (DBI) of erythrocyte ghosts was not significantly altered by dietary supplementation. Supplementation with fish oil significantly increased the n-3/n-6 ratio vs. pre-supplementation values ($P < 0.01$).

Membrane phospholipid content. The calculated phospholipid contents of young and old erythrocyte ghosts prior to and following dietary supplementation with SAF or fish oil are shown in Figure 1. Prior to dietary intervention, there were no significant differences in phospholipid content

TABLE 2
Four-Week Dietary Fat Consumption Patterns Reported Prior to (Pre) and at the Completion (Post) of the Intervention Period[a]

	Safflower (n = 9)		Fish oil (n = 8)	
Daily fat intake	Pre	Post	Pre	Post
Total (% total cal)	32.6 ± 2.7	27.8 ± 2.9	31.8 ± 0.9	31.7 ± 3.2
Saturated (g/d)	24.3 ± 3.6	19.9 ± 3.8	24.3 ± 5.4	19.3 ± 2.9
Monounsaturated (g/d)	24.1 ± 3.3	18.5 ± 2.9	23.8 ± 5.3	19.3 ± 3.0
18:2n-6 (g/d)	9.1 ± 1.7	8.6 ± 1.8	13.3 ± 3.1	8.7 ± 1.6
P/S Ratio	0.4 ± 0.1	0.4 ± 0.03	0.6 ± 0.1	0.5 ± 0.1

[a]Values represent X ± SEM; P/S, polyunsaturated fat/saturated fat.

TABLE 3
Fatty Acid Composition of Erythrocyte Ghosts (μg/mg protein) from Young and Old Cells[a]

	Safflower oil				Fish oil			
	Pre-supplement		Post-supplement		Pre-supplement		Post-supplement	
	Young	Old	Young	Old	Young	Old	Young	Old
14:0	0.4 ± 0.2	2.0 ± 0.5†	0.8 ± 0.6	0.4 ± 0.1*	0.8 ± 0.5	1.9 ± 0.5	0.9 ± 0.6	0.9 ± 0.6
16:0	20.8 ± 3.0	27.3 ± 1.4	28.6 ± 3.6*	27.4 ± 2.6	20.1 ± 1.1	20.2 ± 1.7	26.1 ± 3.4*	27.8 ± 1.6**
18:0	14.6 ± 2.2	17.0 ± 1.6	21.1 ± 3.4*	20.0 ± 2.4	14.2 ± 1.3	14.3 ± 1.5	19.6 ± 3.0	18.7 ± 2.0
18:1n-9	15.8 ± 3.1	17.7 ± 1.5	20.8 ± 2.6*	20.4 ± 2.3	14.0 ± 1.2	13.8 ± 1.3	18.2 ± 2.9	18.2 ± 1.7
18:2n-6	10.8 ± 2.5	11.1 ± 1.1	12.6 ± 1.6	12.4 ± 1.6	8.5 ± 0.8	8.2 ± 0.8	9.5 ± 1.3	10.0 ± 0.9
18:3n-6	0.8 ± 0.5	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	1.6 ± 1.2	0.6 ± 0.1
18:3n-3	1.9 ± 1.5	0.4 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.8 ± 0.4	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.1*
20:3n-6	2.9 ± 0.6	2.8 ± 0.4	3.2 ± 0.5	3.4 ± 0.4	2.3 ± 0.3	2.7 ± 0.3	3.6 ± 1.1	3.3 ± 0.2
20:4n-6	13.8 ± 2.1	15.1 ± 1.3	16.9 ± 2.1	16.0 ± 2.0	12.7 ± 1.3	12.6 ± 1.4	13.4 ± 2.0	15.9 ± 2.2
20:5n-3	0.7 ± 0.2	0.9 ± 0.5	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	1.6 ± 0.3**	1.6 ± 0.2**
22:4n-6	8.9 ± 3.0	7.9 ± 0.6	11.3 ± 2.7	8.7 ± 1.2	6.2 ± 0.9	6.5 ± 0.7	8.0 ± 1.4	8.8 ± 1.0
22:5n-6	0.7 ± 0.2	1.1 ± 0.1	1.5 ± 0.3*	1.1 ± 0.1	0.5 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	1.7 ± 1.0
22:5n-3	2.7 ± 0.6	2.4 ± 0.2	2.9 ± 0.5	2.3 ± 0.4	1.8 ± 0.2	1.8 ± 0.2	2.7 ± 0.4	2.9 ± 0.3**
22:6n-3	3.2 ± 0.6	3.6 ± 0.4	5.5 ± 1.4	3.6 ± 0.5	3.1 ± 0.4	3.4 ± 0.5	4.5 ± 0.7	5.3 ± 0.5*
24:0	2.8 ± 0.5	5.9 ± 1.2	4.2 ± 0.9	3.7 ± 0.5	2.7 ± 0.7	3.3 ± 0.4	3.0 ± 0.7	4.4 ± 0.5
Σn-3	8.5 ± 2.5	7.3 ± 0.9	9.3 ± 1.8	6.9 ± 1.0	6.9 ± 0.9	6.1 ± 0.9	9.1 ± 2.1	10.8 ± 0.9
Σn-6	37.8 ± 2.1	38.6 ± 3.1	46.0 ± 4.7	42.1 ± 5.1	32.6 ± 4.7	30.2 ± 5.1	39.3 ± 9.2	43.7 ± 3.0
n-3/n-6	0.21 ± 0.02	0.19 ± 0.01	0.20 ± 0.03	0.16 ± 0.01	0.22 ± 0.02	0.21 ± 0.02	0.24 ± 0.04	0.25 ± 0.02
DBI	2.88 ± 0.05	2.85 ± 0.02	2.90 ± 0.12	2.77 ± 0.03	2.87 ± 0.05	2.88 ± 0.05	2.89 ± 0.14	2.97 ± 0.05

[a]Separated by density, prior to and following six weeks' dietary supplementation with either safflower or fish oil. DBI = double-bond index. Values represent X ± SEM. *$P < 0.05$ vs. pre-diet; **$P < 0.01$ vs. pre-diet; †$P < 0.05$ vs. young cells.

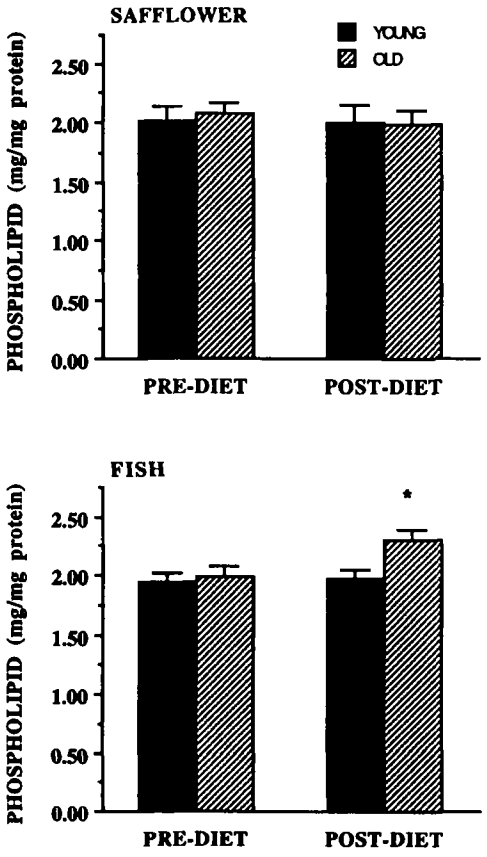


FIG. 1. Calculated phospholipid content (μg/mg protein) of young and old erythrocytes prior to and following six weeks' dietary supplementation with 4.0 g/day safflower oil or fish oil. All values represent X ± SEM. $^*P < 0.05$ vs. young cells.

(mg/mg protein) of either young or old ghosts in either dietary supplementation group. While supplementation with SAF had no effect on phospholipid content in either young or old ghosts, supplementation with fish oil significantly increased the phospholipid content of old ghosts vs. pre-treatment ($P < 0.05$). The phospholipid content of young ghosts, however, was unaffected by fish oil supplementation.

Water content. The water contents of young and old erythrocytes, separated by density centrifugation, prior to and following dietary supplementation with SAF or fish oil are shown in Figure 2. The water content of old erythrocytes (l/kg solids) was significantly lower than that of young erythrocytes ($P < 0.001$) and was unaffected by either dietary fatty acids or time.

Anion exchange rate constant. The rate constant, *k*, of SO_4^{2-} efflux from young and old erythrocytes, prior to and following dietary treatment with n-6 and n-3 fatty acids, is shown in Figure 3. Prior to dietary intervention, the efflux rate constant in both treatment groups was significantly higher in the old cells than in the young cells ($P < 0.01$). This age-related difference was approximately 10.6 and 12.7% in the SAF and fish oil groups, respectively. Following six weeks of dietary supplementation with SAF, there was no change in the efflux constant in either the young or old cells from the pre-treatment

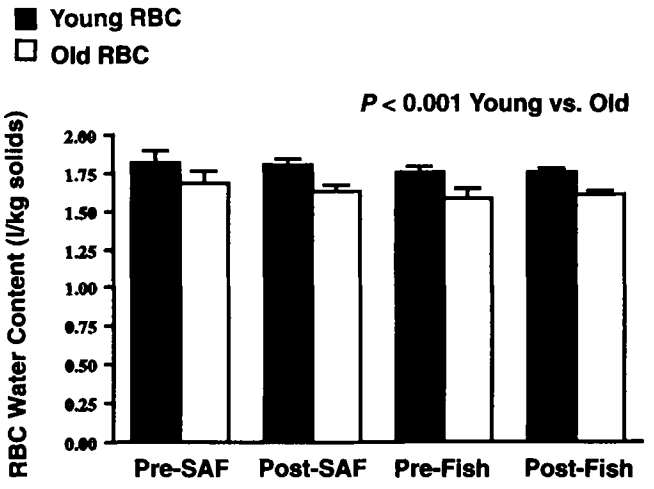


FIG. 2. Erythrocyte water content (1/kg solids) in young and old cells before and after dietary supplementation, as in Figure 1. RBC, red blood cells; SAF, safflower oil.

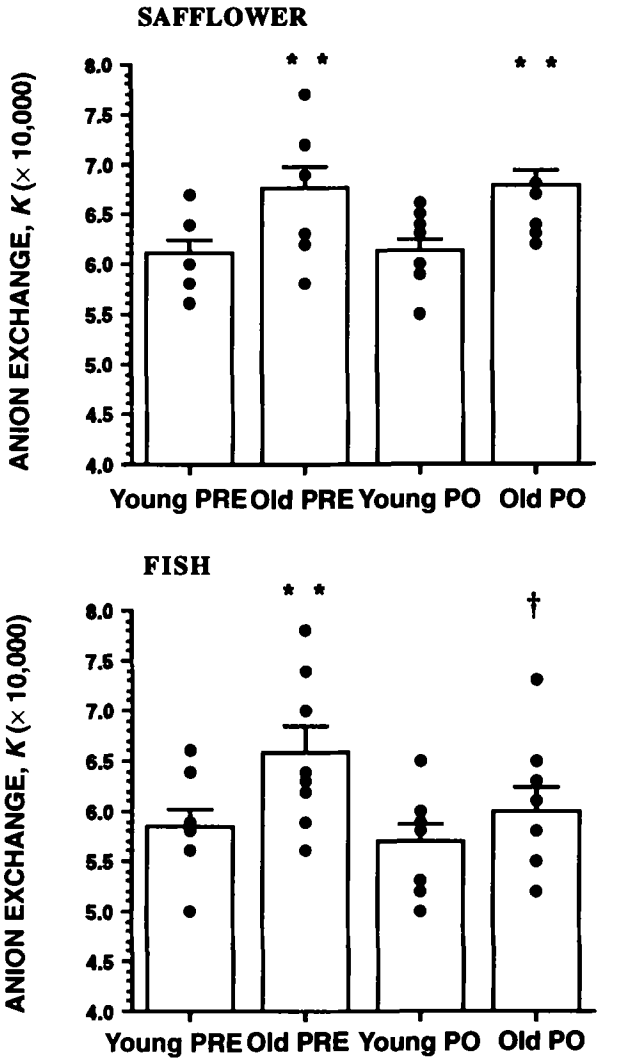


FIG. 3. Anion exchange rate constant, *k*, of young and old erythrocytes prior to (PRE) and following (PO) dietary supplementation, as in Figure 1. $^{**}P < 0.01$ vs. young cells. $^{\dagger}P < 0.05$ vs. pre-diet.

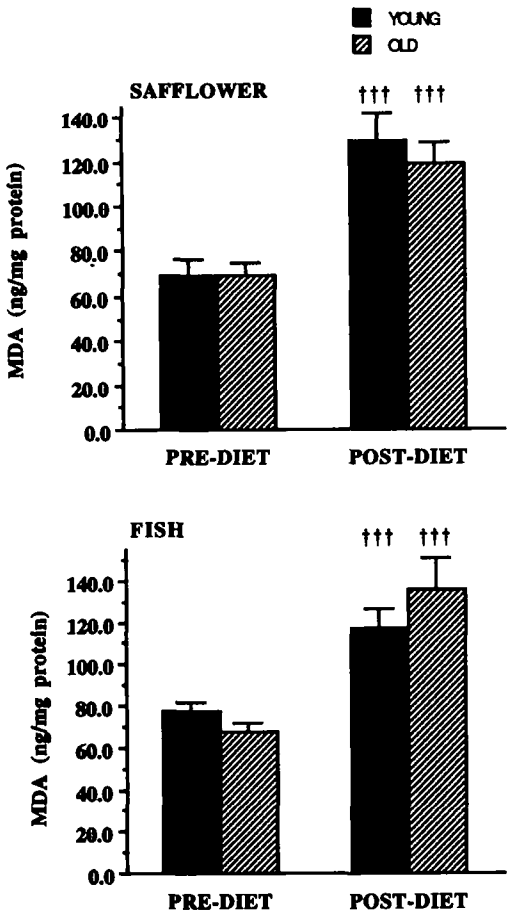


FIG. 4. Thiobarbituric acid-reactive substances production [malondialdehyde (MDA)] in young and old erythrocytes prior to and following dietary supplementation, as in Figure 1. $P < 0.001$ vs. pre-diet.

values, and the k in the old cells was significantly higher than in the young cells ($P < 0.01$). Following fish oil supplementation, however, the efflux constant of the old cells was significantly reduced from the pre-treatment value ($P < 0.02$), so that the efflux constants of the young and old cells were not significantly different from each other. There was no change in the k of the young erythrocytes vs. pre-treatment.

Oxidative sensitivity. The TBARS production in young and old erythrocyte ghosts prior to and following dietary supplementation with SAF and fish oil is shown in Figure 4. Prior to dietary intervention, there were no differences in TBARS production (ng/mg protein) in response to a superoxide generator between young and old erythrocyte ghosts in either treatment group. Following dietary supplementation with either SAF or fish oil, TBARS production was significantly higher in both young and old ghosts ($P < 0.001$) vs. pre-supplementation. However, TBARS production was influenced by neither the erythrocyte age nor the type of dietary fat (SAF or fish oil) used.

DISCUSSION

Cell age-related changes in erythrocyte phospholipid fatty acid composition have been reported by several investigators, although the exact nature of these changes varies among the reports (1–6,26,27). As erythrocyte membrane fatty acids are known to exchange with those in plasma phospholipids over a relatively short period of time (8–12 d) (4), it has been suggested that the differences among studies might be due, in part, to variations in the pattern and quantity of dietary fat intake over the course of the study (4). In the present study, fatty acid composition of erythrocyte ghost total lipids did not exhibit consistent age-related differences, although a decrease in the n-3/n-6 ratio in old cells vs. young cells was observed, as in our previous report (21). The absence of age-related differences in specific fatty acids in the present study may be due in part to (i) the use of the total lipid extract of the erythrocyte ghost in the present study vs. the erythrocyte phospholipid fraction for fatty acid analysis as in previous studies and (ii) the reporting of absolute levels of fatty acids in the present study vs. the reporting of relative levels (percentage of total fatty acids) in previous work.

Dietary supplementation with SAF decreased the n-3/n-6 ratio in old cells and decreased the DBI of both young and old cells. It also led to increased 22:4n-6 in young cells. The decrease in n-6 previously reported following SAF supplementation (21) was not observed in this study. Supplementation with fish oil increased long-chain n-3 fatty acid levels in both young and old cells, as previously reported, and increased the n-3/n-6 ratio of old cells.

Differences among the present study and our previous study with respect to erythrocyte lipid responses to dietary SAF administration may be related to methodological differences between the studies. In the earlier study, dietary supplementation was carried out over 12 wk in contrast to the 6 wk in this study. The question of possible shifts in dietary intake patterns during the study was addressed by the administration of a food frequency questionnaire pre- and post-dietary supplementation. This survey indicated that, aside from the experimental supplement, no significant changes in the intake of total calories, total, saturated, or monounsaturated fat, or linoleic acid occurred in any of the experimental groups during the study. This measure, not used in previous studies of age-related changes in erythrocyte lipids, suggests that the present findings were not attributable to uncontrolled dietary alterations.

The use of centrifugation techniques to separate young and old erythrocytes for comparison prior to and after dietary intervention makes the assumption that the dietary intervention does not alter the relative densities of the young and old cell populations. The validity of this assumption is critical to properly interpreting this and previous studies. Unfortunately, there have been no published investigations that address this issue. Nevertheless, we believe this assumption is valid because in our previous study, supplementation with SAF did not significantly affect several density-dependent cell membrane characteristics normally associated with cell aging, including relative filtration time, anion exchange constants, and water content. Density separation demonstrated the age-re-

lated characteristics similarly in both unsupplemented and safflower-supplemented subjects. Fish oil supplementation differentially affected the measured parameters, suggesting that supplementation *per se* did not alter the relationship.

We have previously reported increases in cell deformability and membrane lipid composition of old erythrocytes following fish oil supplementation. The present data demonstrate a fish oil-related abolition of the age-related increase in erythrocyte membrane anion transport. Together, these reports suggest that the functional changes brought about in the erythrocyte membrane by dietary long chain n-3 fatty acids are quite broad, and may represent a general alteration of the physiology of the aging cell. Whether or not these changes also result in the alteration of the oxygen delivery capability of the old cell or its survival time in the circulation, however, remains to be tested. Furthermore, it also would be of interest to determine whether or not dietary n-3 fatty acids alter other primary aging phenomena, such the expression of senescent cell antigen (8).

While the described changes brought about by dietary fish oil in the aging erythrocyte at first appear to be beneficial, the addition of highly unsaturated fatty acids, such as 20:5n-3 and 22:6n-3 to the erythrocyte membrane, might also increase the burden on cellular antioxidant defense mechanisms and lead to exaggerated oxidative damage. In the present study, both SAF and fish oil supplementation significantly and similarly increased TBARS production by erythrocyte ghosts during *in vitro* incubation with a free radical generator vs. pre-dietary controls. TBARS production was independent of cell age, supplement fatty acid composition, and post-intervention DBI of erythrocyte ghosts. This indicates that fish oil is no more deleterious in this regard than the SAF. Why fish oil, with its greater degree of unsaturation, is not associated with a greater degree of oxidative damage is not clear. It is possible that above a certain dietary fat level, the amount of dietary fat is more critical than the particular type of fat. Alternatively, the consumption of the highly unsaturated fish oil has been shown to lead to a compensatory increase in cellular antioxidant defenses (28), although this is not always observed (29). It is also possible that the *in vitro* reaction produced oxidative products other than MDA, e.g., lipid peroxides, which are not detectable with the TBARS method. Perhaps the most interesting aspect of the latter experiment was that the supplemental dietary 18:2n-6 (~29% above regular diet) had such a powerful effect on the oxidative susceptibility of the erythrocyte.

The current findings lend further support to the concept that dietary supplementation with sources of long-chain n-3 fatty acid sources selectively attenuate a number of age-related membrane changes seen in the aging human erythrocyte. These changes occur in the apparent absence of an increased susceptibility of the membrane lipids to oxidative damage. The potential benefits of these dietary-induced changes on erythrocyte function *in vivo* remain to be seen.

ACKNOWLEDGMENTS

This work was conducted with the support of grants-in-aid from the Heart and Stroke Foundation of Ontario and the Natural Sciences and Engineering Research Council of Canada to D. Mills and M. Murthy, and from the American Heart Association (GNM-04-90) to W.R. Galey. The authors would like to thank the Efamol Research Institute, Kentville, Nova Scotia, for donating the fish oil (Polepa) and safflower oil for the study.

REFERENCES

1. Munn, J.I. (1958) Studies of Lipids in Human Red Cells, *Brit. J. Haematol. 4*, 34–39.
2. Westerman, M., Pierce, L.E., and Jensen, J. (1963) Erythrocyte Lipids: A Comparison of Normal Young and Normal Old Populations, *J. Lab. Clin. Med. 62*, 394–400.
3. Van Gastel, C., Van Den Berg, D., De Gier, J., and Van Deenan, L.M. (1965) Some Lipid Characteristics of Normal Red Blood Cells of Different Ages, *Brit. J. Haematol. 11*, 193–199.
4. Walker, B.L., and Yurkowski, M. (1967) Effect of Cell Age on Erythrocyte Fatty Acid Composition in Rats on Different Dietary Regimes, *Biochem. J. 108*, 218–224.
5. Phillips, G.B., Dodge, J.T., and Howe, C. (1970) The Effect of Aging of Human Red Cells *in Vivo* on Their Fatty Acid Composition, *Lipids 4*, 544–549.
6. Kunimoto, M., Kaya, K., and Miura, T. (1984) Composition and Metabolism of Fatty Acids in Phospholipids of Density-Separated Red Cells of Rats, *Lipids 19*, 443–448.
7. Zanner, M.A., and Galey, W.R. (1985) Aged Human Erythrocytes Exhibit Increased Anion Exchange, *Biochim. Biophys. Acta 818*, 310–315.
8. Kay, M.M.B. (1981) Isolation of the Phagocytosis-Inducing IgG-Binding Antigen on Senescent Somatic Cells, *Nature 289*, 491–494.
9. Pfeffer, S.R., and Swislocki, N.I. (1976) Age-Related Decline in the Activities of Erythrocyte Membrane Adenylate Cyclase and Protein Kinase, *Arch. Biochem. Biophys. 177*, 117–122.
10. Weed, R.I., Lacelle, L., and Merrill, E.W. (1969) Metabolic Dependence of Red Cell Deformability, *J. Clin. Invest. 48*, 795–809.
11. Marks, P.A., and Johnson, A.B. (1958) Relationship Between the Age of Human Erythrocytes and Their Osmotic Resistance: A Basis for Separating Young and Old Erythrocytes, *J. Clin. Invest. 37*, 1542–1548.
12. Terano, T., Hirai, A., Hamazaki, T., Kobayashi, S., Fujita, T., Tamura,Y., and Kumagai, A. (1983) Effect of Oral Administration of Highly Purified Eicosapentaenoic Acid on Platelet Function, Blood Viscosity and Red Cell Deformability in Healthy Human Subjects, *Atherosclerosis 46*, 321–331.
13. Popp-Snijders, C., Schouten, J.A., DeJong, A.P., and Van Der Veen, E.A. (1984) Effect of Dietary Cod-Liver Oil on the Lipid Composition of Human Erythrocyte Membranes, *Scand. J. Lab. Clin. Invest. 44*, 39–46.
14. Tamura, Y., Hirai, A., Terano, T., Tahara, K., Yoshida, S., and Kumagai, A. (1984) Effect of Administration of Highly Purified Eicosapentaenoic Acid on Platelet and Erythrocyte Functions in Patients with Thrombotic Disorders, in *Nutritional Prevention of Cardiovascular Disease*, pp. 323–330, Academic Press, New York.
15. Cartwright, I.J., Pockley, A.G., Galloway, J.H., Greaves, M., and Preston, F.E. (1985) The Effects of Dietary ω-3 Polyunsaturated Fatty Acids and Erythrocyte Membrane Phospholipids, Erythrocyte Deformabilty and Blood Viscosity in Healthy Volunteers, *Atherosclerosis 55*, 267–281.
16. Popp-Snijders, C., Schouten, J.A., van der Meer, J., and van der Veen, E.A. (1986) Fatty Fish-Induced Changes in Membrane Lipid Composition and Viscosity of Human Erythrocytes, *Scand. J. Clin. Lab. Invest. 46*, 253–258.
17. Popp-Snijders, C., Schouten, J.A., Blitterswijk, W.J., and van der Veen, E.A. (1986) Changes in Membrane Lipid Composi-

tion of Human Erythrocytes After Dietary Supplementation of (n-3) Polyunsaturated Fatty Acids. Maintenance of Membrane Fluidity, *Biochim. Biophys. Acta 854*, 31–37.
18. Rogers, S., James, K.S., Butland, B.K., Etherington, M.D., O'Brien, J.R., and Jones, J.G. (1987) Effects of a Fish Oil Supplement on Serum Lipids, Blood Pressure, Bleeding Time, Haemostatic and Rheological Variables, *Atherosclerosis 63*, 137–143.
19. Bruckner, G., Webb, P., Greenwell, L., Chow, C., and Richardson, D. (1987) Fish Oil Increases Peripheral Capillary Blood Flow Velocity in Humans, *Atherosclerosis 66*, 237–245.
20. Driss, F., Darcet, PH., Delhaye, N., and Mendy, F. (1988) Effect of Eicosapentaenoic Acid on RBC Filterability and Fatty Acid Composition, *Clin. Hemorheol. 8*, 679–685.
21. Mills, D.E., Galey, W.R., and Dixon, H. (1993) Effects of Dietary Fatty-Acid Supplementation on Fatty Acid Composition and Deformability of Young and Old Erythrocytes, *Biochim. Biophys. Acta 1149*, 313–318.
22. Murphy, J.R. (1973) Influence of Temperature and Method of Centrifugation on the Separation of Erythrocytes, *J. Lab. Clin. Med. 82*, 334–341.
23. Mishell, B.B., and Shiigi, S.M. (1980) in *Selected Methods in Cellular Immunology*, pp. 14–16, W.H. Freeman, San Francisco.
24. Steck, T.L. (1974) in *Methods in Membrane Biology* (Korn, E.D., ed.), Vol. 2, pp. 245–281, Plenum Press, New York.
25. Mills, D.E., Huang, Y-S., and Ward, R.P. (1990) Fatty Acid Metabolism in Normotensive and Hypertensive Rats I. Differential Incorporation of Dietary n-3 and n-6 Fatty Acids, *Nutr. Res. 10*, 663–674.
26. de Gier, J., van Deenen, L.L.M., Verloop, M.C., and van Gastel, C. (1964) Phospholipid and Fatty Acid Characteristics of Erythrocytes in Some Cases of Anaemia, *Brit. J. Haematol. 10*, 246–256.
27. Ways, P., Reed, C.F., and Hanahan, D.J. (1963) Red-Cell and Plasma Lipids in Acanthocytosis. *J. Clin. Invest. 42*, 1248–1260.
28. Demoz, A., Willumsen, N., and Berge, K. (1992) Eicosapentaenoic Acid at Hypotriglyceridemic Dose Enhances the Hepatic Antioxidant Defense in Mice, *Lipids 27*, 968–971.
29. Javouhey-Donzel, A., Guenot, L., Maupoil, V., Rochette, L., and Rocquelin, G. (1993) Rat Vitamin E Status and Heart Lipid Peroxidation: Effect of Dietary α-Linolenic Acid and Marine n-3 Fatty Acids, *Lipids 28*, 651–655.

[Received October 12, 1994, and in revised form March 15, 1995; Revision accepted May 5, 1995]

Separation of γ- and α-Linolenic Acid Containing Triacylglycerols by Capillary Supercritical Fluid Chromatography

Pekka Manninen*, Päivi Laakso and Heikki Kallio

Department of Biochemistry and Food Chemistry, University of Turku, 20500 Turku, Finland

ABSTRACT: The separation of γ- and α-linolenic acid containing triacylglycerols with an identical acyl carbon number and degree of unsaturation was obtained on capillary supercritical fluid chromatography using a 25% cyanopropyl–75% methylpolysiloxane stationary phase. The resolution of 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol and 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol was 1.35 on a 10 m × 50 μm i.d. column, whereas the resolution was enhanced to 1.66 by combining two 10-meter columns in series. The difference in the position of double bonds in one linolenic acid residue of triacylglycerols resulted in two series of peaks in the separation of alpine currant *(Ribes alpinum)* and black currant *(R. nigrum)* seed oils. The use of the 10-meter column was found to be appropriate for the screening of the triacylglycerol profile in both seed oils studied.
Lipids 30, 665–671 (1995).

Edible oils containing γ-linolenic acid, 18:3n-6, are of physiological and nutritional importance. It has been considered that γ-linolenic acid should be regarded as an n-6 essential fatty acid having a notable role in health and disease, as recently reviewed by Horrobin (1). The most important functions of the n-6 essential fatty acids are related to the modulation of membrane structure and the formation of eicosanoids (1). The occurrence, as well as the physical and chemical properties of γ-linolenic acid, are well reviewed by Gunstone (2).

Of the several seed oils containing γ-linolenic acid known today (3), evening primrose (*Oenothera biennis*) oil and borage oil (*Borago officinalis*) are the most well-known. Such oils also have value as commercial products and are consequently under study. Usually the amount of γ-linolenic acid is measured by analyzing the fatty acid composition of the corresponding oil by gas chromatography, as their methyl esters. However, more detailed data of the triacylglycerol composition of edible oils is needed, e.g., to evaluate their nutritional importance and to improve their technological properties.

Using gas chromatography, it has not been possible to separate triacylglycerols with an identical acyl carbon number (ACN) and degree of unsaturation containing either γ- or α-linolenic acid, as reported by Řezanka and Mareš (4). Some articles have been published in which high-performance liquid chromatography (HPLC) has been applied to the separation of triacylglycerols containing γ-linolenic acid residue (5–9). These studies are mostly concerning evening primrose oil and borage oil, in which triacylglycerol compositions were analyzed by silver-ion or reversed-phase HPLC (5,6). However, these oils contain none or very small amounts of α-linolenic acid. Similar separation of triacylglycerols containing γ-linolenic acid also has been reported from the seeds of *Anemone* species by reversed-phase HPLC (7). Perrin *et al.* (8) separated triacylglycerols in black currant seed oil having identical ACNs and degree of unsaturation, containing γ- and α-linolenic acid, with good resolution using two reversed-phase columns in series. Recently, Aitzetmüller and Grönheim (9) produced a similar separation using a 25-cm reversed-phase column for *Primula florindae* and *Ribes nigrum.* Due to the dual nature of the separation mechanism on reversed-phase HPLC, using silver-ion HPLC to separate triacylglycerols according to the degree of unsaturation only has been recommended (5). Furthermore, it may be advantageous to use a combination of silver-ion and reversed-phase HPLC for the analysis of γ-linolenic acid oils.

Supercritical fluid chromatography (SFC) with a packed capillary column impregnated with silver nitrate has been applied to the separation of triacylglycerols in borage oil (10). This technique was able to produce partial separation of γ-linolenoyl-linoleoyl-palmitoyl-glycerol and α-linolenoyl-linoleoyl-palmitoyl-glycerol triacylglycerols. However, no articles of similar separations with open-tubular capillary columns have been published, although capillary SFC has been shown to be able to separate free fatty acids according to the degree of unsaturation (11).

In addition, fatty acids also can be separated as methyl esters by capillary SFC, as shown in this study. Triacylglycerols have been separated according to the ACN and the degree of unsaturation by capillary SFC by using a 25% cyanopropyl–75% methylpolysiloxane stationary phase, and the general elution behavior of triacylglycerols on this particular column has been discussed in more detail in a concurrent pub-

*To whom correspondence should be addressed at Department of Biochemistry and Food Chemistry, University of Turku, Vatselankatu 2, 20500 Turku, Finland.

Abbreviations: ACN, acyl carbon number; c.v., coefficient of variation; HPLC, high-performance liquid chromatography; MS, mass spectrometry; SFC, supercritical fluid chromatography.

Copyright © 1995 by AOCS Press

lication (Manninen, P., Laakso, P., and Kallio, H., unpublished data). In this study, a column with the same stationary phase was used to separate triacylglycerols containing α- and γ-linolenic acid residues. The separation was studied on both 10- and 20-meter columns. The method was applied for the analysis of alpine currant and black currant seed oils.

EXPERIMENTAL PROCEDURES

Materials. The reference compounds 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol (*sn*-18:1n-9-18:3n-6-18:1n-9) and 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol (*sn*-18:1n-9-18:3n-3-18:1n-9) (custom synthesized by Larodan, Malmö, Sweden) were diluted in *n*-hexane (Rathburn Chemical Ltd., Walkerburn, Scotland) to a concentration of approximately 0.5 mg mL^{-1}, and 1 mg of the mixture of trilinoleoyl-glycerol (18:2n-6-18:2n-6-18:2n-6), dilinoleoyl-γ-linolenoyl-glycerol (18:2n-6-18:2n-6-18:3n-6), and linoleoyl-di-γ-linolenoyl-glycerol (18:2n-6-18:3n-6-18:3n-6) (kindly donated by Dr. David Horrobin of Scotia Pharmaceuticals, Guilford, England) was diluted in 1 mL of dichloromethane (Merck, Darmstadt, Germany) for SFC analysis. Alpine currant (*Ribes alpinum)* seed oil and sea buckthorn (*Hippophaë rhamnoides*) seed oil were extracted with supercritical carbon dioxide at Flavex (Rehlingen, Germany) using production scale plant, and black currant (*R. nigrum)* seed oil was isolated by extraction using methanol/chloroform (1:2, vol/vol). The oils were stored under nitrogen below 0°C, and diluted 1:23 by volume in dichloromethane before chromatography. The fatty acid methyl esters were prepared by sodium methoxide catalyzed transesterification of 40 mg of alpine currant and sea buckthorn seed oils (12). The unpurified fatty acid methyl esters were diluted in 0.7 mL dichloromethane before SFC.

Methods. SFC was obtained on a Lee Scientific Series 600 supercritical fluid chromatograph (Lee Scientific, Dionex, Salt Lake City, UT) equipped with a flame-ionization detector and a pneumatically and electrically-controlled Valco switching valve with an internal loop volume of 1.0 μL. The flame-ionization detector was held at the temperature of 340°C, and nitrogen was used as a make-up gas. Frit restrictors (30 cm × 50 μm i.d.; Lee Scientific, Dionex) were connected at the split outlet and to the end of the analytical column. Sample introduction was performed by a combined timed and dynamic split technique. The columns used were: 10 m × 50 μm i.d. SB-Octyl-50 (50% octyl–50% methylpolysiloxane; Lee Scientific, Dionex) with a film thickness of 0.25 μm, and two 10 m × 50 μm i.d. SB-Cyanopropyl-25 (25% cyanopropyl–75% methylpolysiloxane; Lee Scientific, Dionex) with a film thickness of 0.25 μm. A 1/16" zero dead volume butt connector (SGE, Austin, TX) was used to combine two columns in series. SFC-grade CO_2 (Scott Specialty Gases, Plumsteadville, PA) was used as the carrier fluid. The column flow rate of CO_2 was 0.37 mL min^{-1} with the columns of 10 meters and 0.17 mL min^{-1} with the combination of the two 10-meter columns, as measured with propane at a column temperature of 140°C and CO_2 density of 0.140 g mL^{-1}. Chromatography on the 10-meter columns was performed at the constant temperature of 140°C using a linear density ramp from 0.140 g mL^{-1} at a rate of 0.005 g mL^{-1} min^{-1}, unless otherwise mentioned. With the combination of the two 10-meter columns, the linear density ramp used was from 0.150 g mL^{-1} using a 0.004 g mL^{-1} min^{-1} programming rate. Mass spectrometric analyses were obtained on a Finnigan MAT (San Jose, CA) TSQ-700 triple quadrupole mass spectrometer using a direct exposure probe for sample introduction. Ammonia negative-ion chemical ionization with an ammonia pressure of 8500 mtorr and a temperature of 200°C was used to produce the mass spectra of triacylglycerols (13).

RESULTS AND DISCUSSION

The main components in both alpine currant and black currant seed oils, according to the ACN group separation on a nonpolar stationary phase, were triacylglycerols containing 54 acyl carbons (Fig. 1). In addition, the composition of triacylglycerols obtained on mass spectrometry (MS) analysis was very similar, with only minor differences, in both oils (Table 1). The amount of γ-linolenic acid in alpine currant and black currant seed oils studied were 11.5 and 14.7% in weight, respectively, as measured by methyl esters *via* gas

TABLE 1
The Proportions of Triacylglycerols in Alpine Currant and Black Currant Seed Oil Analyzed Using Ammonia Negative-Ion Chemical Ionization Mass Spectrometry

	Alpine currant seed oil		Black currant seed oil	
Triacylglycerol	Proportion[a,b] (%)	SD	Proportion[a,b] (%)	SD
52:7	0.71	0.03	0.64	0.02
52:6	2.57	0.10	2.89	0.11
52:5	5.19	0.12	7.10	0.17
52:4	5.74	0.30	8.54	0.24
52:3	2.43	0.25	3.10	0.18
52:2	0.89	0.09	0.71	0.04
54:10	1.37	0.06	0.81	0.03
54:9	5.46	0.23	3.97	0.08
54:8	13.33	0.47	11.66	0.18
54:7	19.41	0.67	19.60	0.32
54:6	17.82	0.82	19.18	0.28
54:5	12.21	0.30	10.79	0.19
54:4	6.25	0.24	4.81	0.10
54:3	2.20	0.15	1.36	0.04
54:2	0.39	0.04		
56:7			0.48	0.03
56:6			0.80	0.03
56:5	0.37	0.04	0.81	0.02
56:4	0.58	0.07	0.43	0.02
56:3	0.78	0.12		
56:2	0.71	0.11		
56:1	0.45	0.09		
Trace[c]	1.14	0.15	2.38	0.26

[a]The proportions of triacylglycerols are calculated according to the ^{13}C-corrected intensities of $[M - H]^-$ ions.
[b]Four replicates.
[c]The combined proportion of components corresponding to less than 0.3% of the total.

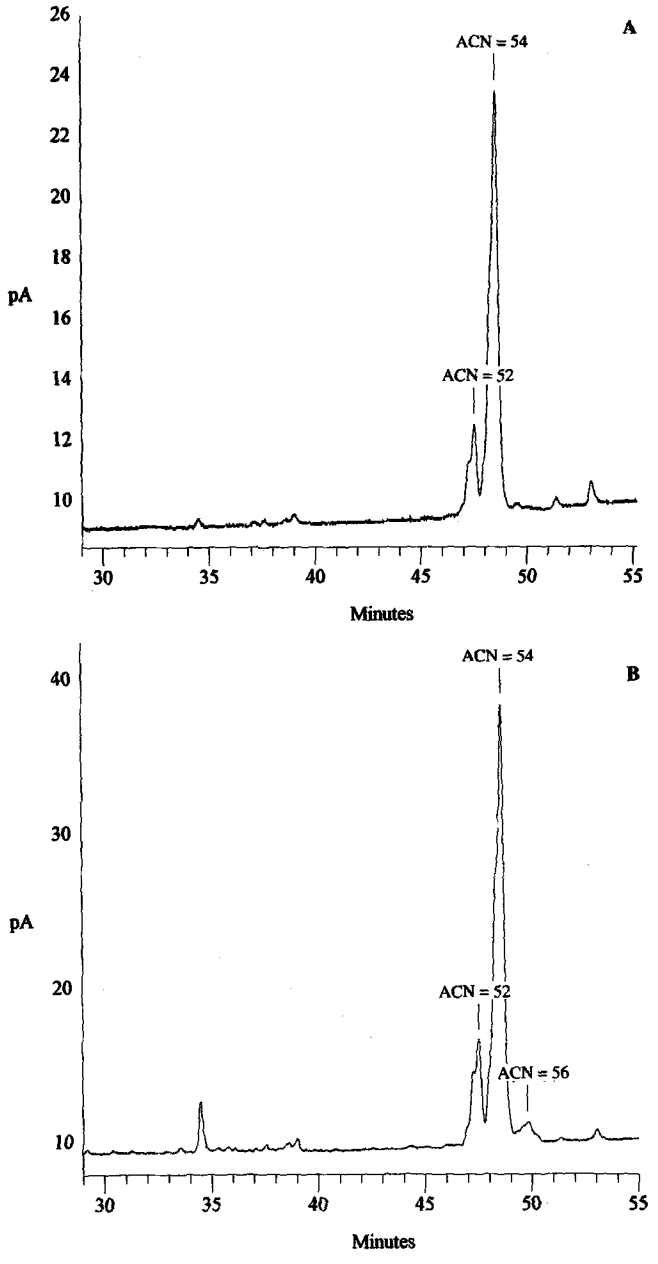


FIG. 1. Separation of alpine currant and black currant seed oils on an SB-Octyl-50 column (Lee Scientific, Dionex, Salt Lake City, UT): (A) alpine currant seed oil, and (B) black currant seed oil. Loop open time 0.7 s. ACN, acyl carbon number.

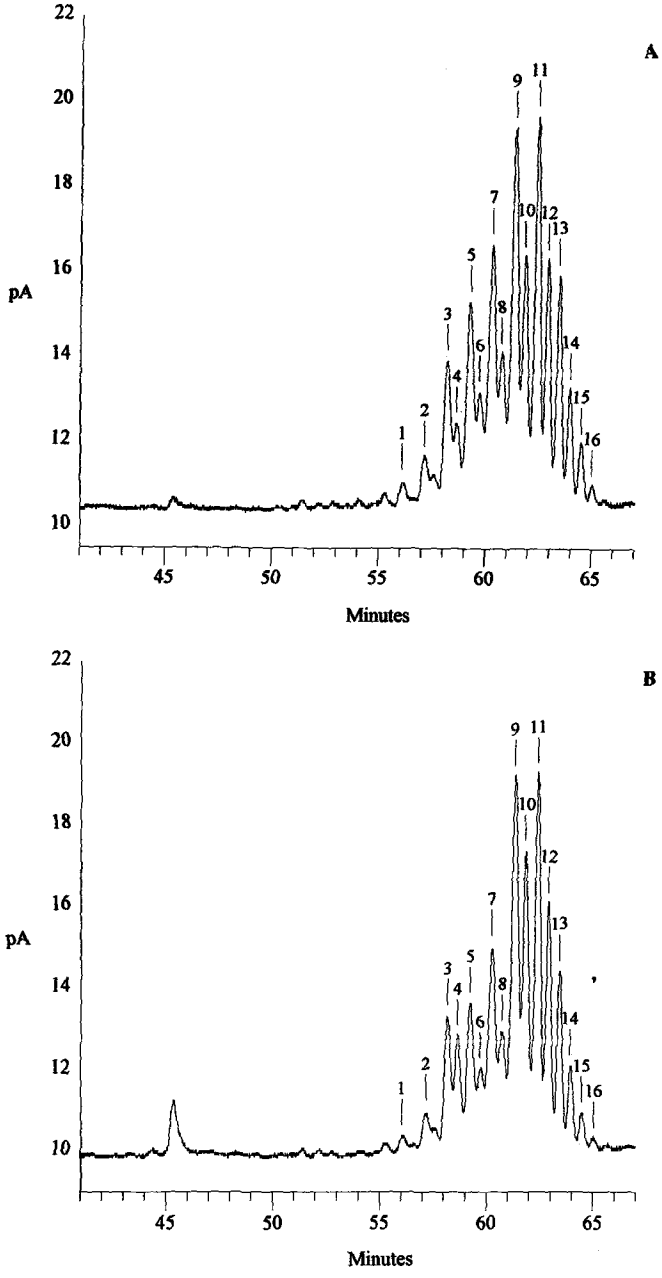


FIG. 2. Separation of triacylglycerols on an SB-Cyanopropyl-25 column (10 m × 50 μm i.d.; Lee Scientific, Dionex): (A) alpine currant seed oil, and (B) black currant seed oil. For peak identification see Table 2. See Figure 1 for company's location.

chromatography. The content of α-linolenic acid was found to be 18.3% by weight in alpine currant seed oil and 14.6% by weight in black currant seed oil fatty acids.

The separation of alpine currant and black currant seed oils on an SB-Cyanopropyl-25 column resulted in two series of prominent peaks (Fig. 2). This chromatographic resolution was not observed in the earlier study of sea buckthorn pulp and seed oil, and cloudberry seed oil (Manninen, P., Laakso, P., and Kallio, H., unpublished data). Such oils do not contain any γ-linolenic acid. The difference in the retention of triacylglycerols containing γ-linolenic acid from those containing α-linolenic acid or no 18:3 fatty acid residues could explain the additional series of triacylglycerol peaks in alpine currant and black currant seed oils. In addition, both alpine currant seed oil and black currant seed oil contained a small amount of stearidonic acid, 18:4n-3, which also could have influenced the resultant separation.

The retention behavior of triacylglycerols containing either γ- or α-linolenic acid could be predicted to some extent by studying the elution pattern of their fatty acid methyl esters. Triacylglycerols do not contain any free carboxylic acid group; therefore, it was more reasonable to study methyl ester derivatives than free fatty acids. The methyl esters of fatty acids of sea buckthorn oil were used to confirm the elution

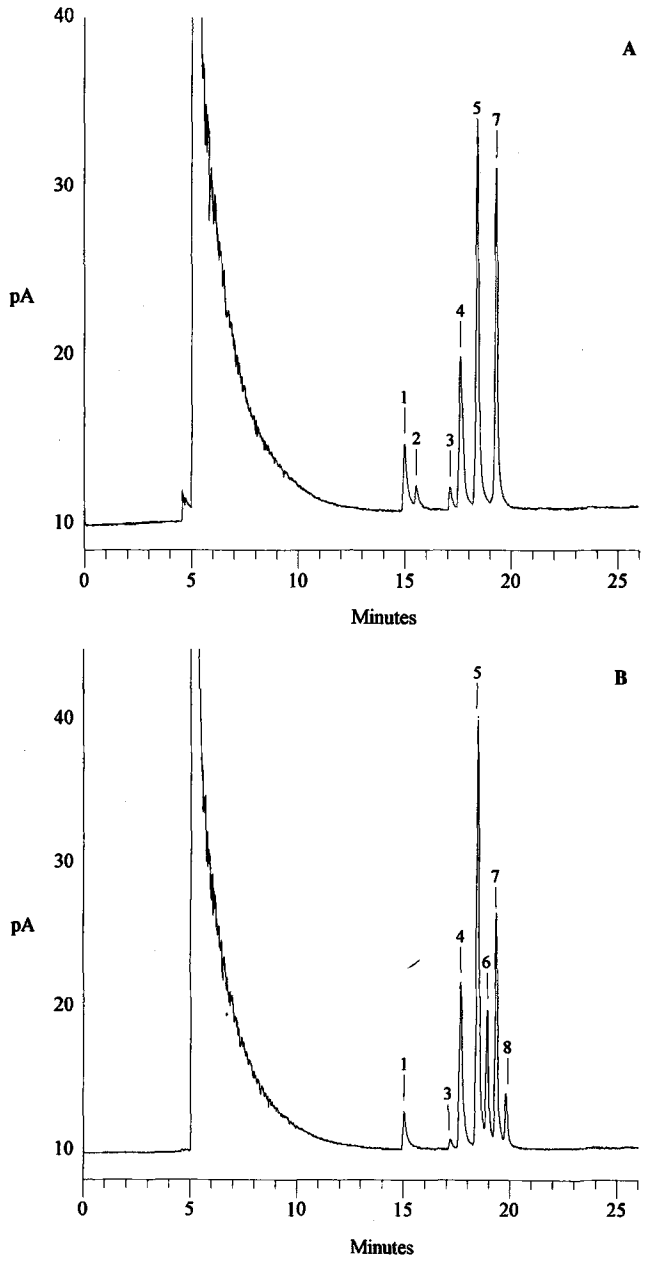


FIG. 3. Separation of fatty acid methyl esters on an SB-Cyanopropyl-25 column (10 m × 50 μm i.d.; Lee Scientific, Dionex): (A) sea buckthorn seed oil and (B) alpine currant seed oil. Chromatographic conditions: linear density ramp from 0.16 g mL^{-1} with 0.010 g $mL^{-1}min^{-1}$. Peak identification: 1 = 16:0, 2 = 16:1n-7, 3 = 18:0, 4 = 18:1n-9, 5 = 18:2n-6, 6 = 18:3n-6, 7 = 18:3n-3, and 8 = 18:4n-3. See Figure 1 for company's location.

order of the fatty acid methyl esters of alpine currant seed oil (Fig. 3). The retention of the fatty acid methyl esters increased with the increasing number of double bonds. In addition, the methyl ester of γ-linolenic acid eluted before that of α-linolenic acid, which indicates that the closer the double bonds are positioned to the ester group, the weaker their interaction between the stationary phase. Therefore, it was reasonable to assume that triacylglycerols with identical ACN and degree of unsaturation, containing either γ- or α-linolenic acid residues, could have slightly different retention.

A two-component mixture containing *sn*-18:1n-9-18:3n-6-18:1n-9 and *sn*-18:1n-9-18:3n-3-18:1n-9 was used to confirm this assumption (Fig. 4A). The component containing the γ-isomer of linolenic acid eluted before that containing the α-isomer, with a resolution of 1.35. The elution behavior was similar to that observed with fatty acid methyl esters. A three-component mixture containing 18:2n-6-18:2n-6-18:2n-6, 18:2n-6-18:2n-6-18:3n-6, and 18:2n-6-18:3n-6-18:3n-6 was separated with the resolutions (R) of $R_{3,4} = 1.12$ and $R_{4,5} = 1.46$ (Fig. 4B). *sn*-18:1n-9-18:3n-6-18:1n-9 and *sn*-18:1n-9-18:3n-3-18:1n-9 were spiked in alpine currant seed oil to confirm the elution order of triacylglycerols with identical ACN and degree of unsaturation having either one γ-linolenic acid or α-linolenic acid moiety. Spiking a real sample with the three-component mixture revealed that triacylglycerols containing two γ-linolenic acid moieties co-eluted with triacylglycerols having identical ACN and one less double bond without any γ-linolenic acid; for example, 54:7 with two γ-linolenic acid moieties co-eluted with 54:6 with only α-isomer of linolenic acid or with no linolenic acid moieties. The difference in the resolution of triacylglycerols containing two α-linolenic acid residues and those with one α- and one γ-linolenic acid was expected to be similar to that of triacylglycerols containing either one α- or one γ-linolenic acid moiety.

The separations were also tested with a longer column (Figs. 4C and 4D). Two 10-meter columns were combined *via* a zero dead volume SGE butt connector, because 20 m × 50 μm i.d. SB-Cyanopropyl-25 columns were, unfortunately, no longer available. The resolution of 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol and 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol was enhanced to 1.66 (Fig. 4C). Similarly, the resolution of the three component mixture increased to $R_{3,4} = 1.67$ and $R_{4,5} = 1.46$ (Fig. 4D).

The analysis of alpine currant and black currant seed oils on a 20-meter column improved the separation of the early eluted peaks (Fig. 5). Although the resolution was enhanced and chromatographic conditions further optimized, very similar proportions of the peaks were obtained with both lengths of the column (Table 2). The repeatability of the proportions of triacylglycerols was good in both cases. However, the repeatability in retention times was not satisfactory using the 20-meter column. It would be appropriate to use the 10-meter column for screening the triacylglycerol profile of both oils studied, even though the two peaks could not be effectively integrated using the 10-meter column.

The proportion of the peaks of triacylglycerols containing one γ-linolenic acid was 35.0% in alpine currant seed oil, which was in agreement with the proportion of γ-isomer of linolenic acid (38.6%). In black currant seed oil, the proportion of corresponding peaks was 37.7%, whereas the proportion of γ-isomer of the linolenic acid was 50.1%. Both alpine currant seed oil and black currant seed oil also contained stearidonic acid, 18:4n-3, 4.4 and 3.2% by weight, respectively. The effect of stearidonic acid to the retention of tria-

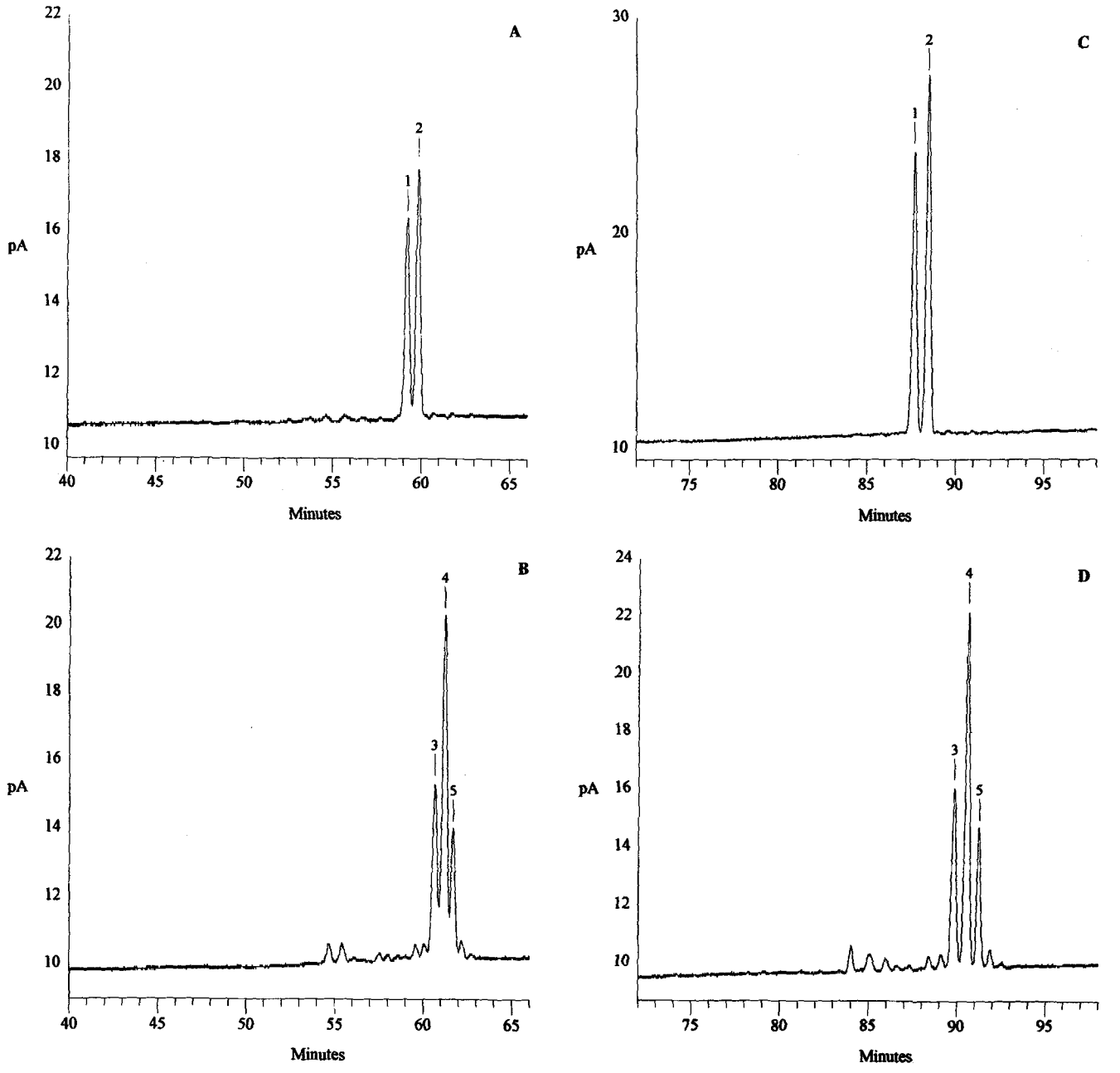


FIG. 4. The separation of reference mixtures of triacylglycerols on an SB-Cyanopropyl-25 column (Lee Scientific, Dionex): (A) a two-component mixture on 10 m x 50 μm i.d. column, (B) a three-component mixture on 10 m x 50 μm i.d. column, (C) a two-component mixture on 20 m x 50 μm i.d. column, and (D) a three-component mixture on 20 m x 50 μm i.d. column. Peak identification: 1-sn-18:1n-9-18:3n-6-18:1n-9, 2 = sn-18:1n-9-18:3n-3-18:1n-9, 3 = 18:2n-6-18:2n-6-18:2n-6, 4 = 18:2n-6-18:2n-6-18:3n-6, and 5 = 18:2n-6-18:3n-6-18:3n-6. See Figure 1 for company's location.

cylglycerols could not be examined during this study because of the lack of reference compounds. However, the difference between the proportion of triacylglycerols containing γ-linolenic acid in black currant seed oil expected and that actually observed (according to the proportion of γ-isomer of the linolenic acid) could not be explained by the amount of stearidonic acid alone, because it was too low. However, the greater difference in proportion indicates that black currant seed oil may contain triacylglycerols having two γ-linolenic acid residues. The presence of such triacylglycerols in black currant seed oil has been reported by Perrin *et al.* (8) and Aitzetmüller and Grönheim (9).

The most abundant triacylglycerols in alpine currant and black currant seed oils based on the MS analysis were 54:5, 54:6, 54:7, and 54:8. The distribution of triacylglycerols containing one γ-linolenic acid residue was quite similar in both oils studied. Among isomeric triacylglycerols (equal number of acyl carbons and double bonds), the proportion of triacylglycerols containing one γ-linolenic acid residue increased with increasing degree of unsaturation. However, one notable

TABLE 2
The Retention Times and the Proportions of Triacylglycerols in Alpine Currant and Black Currant Seed Oils Using 10- and 20-Meter SB-Cyanopropyl-25 Columns[a]

Peak no.	Compound[b] major/minor(s)[c]	Alpine currant seed oil (10 m × 50 μm i.d. column) t_R[d] (min)	SD	Proportion (%)	SD	c.v.[e] (%)	Alpine currant seed oil (20 m × 50 μm i.d. column) t_R[d] (min)	SD	Proportion (%)	SD	c.v.[e] (%)
1	52:2	56.15	0.09	1.03	0.08	8.14	83.38	0.26	0.73	0.07	9.73
2	52:3 (γ)	not integrated					84.00	0.21	0.49	0.03	5.20
3	52:3/54:2,56:1	57.17	0.07	2.11	0.09	4.04	84.66	0.25	1.95	0.06	3.02
4	52:4/54:3 (γ)	not integrated					85.28	0.24	1.33	0.06	4.73
5	52:4/54:3,56:2	58.22	0.07	6.18	0.11	1.85	86.02	0.26	6.08	0.06	0.96
6	54:4/52:5,56:3 (γ)	58.69	0.08	2.98	0.08	2.76	86.70	0.25	2.89	0.10	3.62
7	54:4/52:5,56:3	59.27	0.08	8.68	0.17	2.02	87.36	0.29	8.59	0.10	1.16
8	54:5/52:6,56:4 (γ)	59.76	0.08	4.01	0.22	5.52	88.04	0.31	4.00	0.12	2.92
9	54:5/52:6,56:4	60.30	0.07	11.09	0.11	1.01	88.64	0.36	10.71	0.10	0.90
10	54:6/52:7,56:5 (γ)	60.77	0.06	5.75	0.09	1.51	89.32	0.31	5.60	0.06	1.14
11	54:6/52:7,56:5	61.35	0.08	14.85	0.16	1.06	90.07	0.33	14.59	0.13	0.88
12	54:7 (γ)	61.84	0.07	8.67	0.12	1.37	90.71	0.31	8.61	0.08	0.94
13	54:7	62.41	0.08	13.49	0.09	0.68	91.44	0.33	13.24	0.08	0.64
14	54:8 (γ)	62.92	0.07	7.83	0.06	0.77	92.10	0.32	7.86	0.09	1.11
15	54:8	63.45	0.07	7.29	0.06	0.80	92.76	0.32	7.27	0.15	2.13
16	54:9 (γ)	63.97	0.07	3.56	0.07	2.08	93.43	0.29	3.60	0.07	2.00
17	54:9	64.52	0.06	1.89	0.05	2.58	94.08	0.29	1.90	0.10	5.22
18	54:10 (γ)	65.07	0.07	0.58	0.03	4.30	94.76	0.28	0.62	0.05	8.08

Peak no.	Compound[b] major/minor(s)[c]	Black currant seed oil (10 m × 50 μm i.d. column) t_R[d] (min)	SD	Proportion (%)	SD	c.v.[e] (%)	Black currant seed oil (20 m × 50 μm i.d. column) t_R[d] (min)	SD	Proportion (%)	SD	c.v.[e] (%)
1	52:2	56.02	0.03	0.70	0.11	15.19	83.43	0.48	0.49	0.05	9.25
2	52:3 (γ)	not integrated					83.99	0.46	0.44	0.07	14.92
3	52:3	57.09	0.05	1.79	0.11	6.34	84.72	0.48	1.85	0.28	15.25
4	52:4/54:3 (γ)	not integrated					85.24	0.47	1.22	0.15	12.41
5	52:4/54:3	58.13	0.04	5.83	0.10	1.73	86.08	0.48	5.90	0.10	1.73
6	54:4/52:5 (γ)	58.61	0.05	4.61	0.07	1.57	86.74	0.48	4.28	0.13	3.15
7	54:4/52:5	59.19	0.05	6.91	0.14	1.96	87.42	0.47	7.01	0.12	1.71
8	54:5/52:6,56:4 (γ)	59.70	0.04	3.28	0.05	1.49	88.10	0.47	3.67	0.25	6.83
9	54:5/52:6,56:4	60.20	0.05	9.70	0.11	1.09	88.64	0.51	8.94	0.14	1.54
10	54:6/52:7,56:5 (γ)	60.68	0.06	4.84	0.10	2.06	89.29	0.49	4.89	0.08	1.57
11	54:6/52:7,56:5	61.26	0.07	16.75	0.19	1.11	90.04	0.51	16.62	0.29	1.77
12	54:7/56:6 (γ)	61.77	0.06	11.62	0.14	1.20	90.70	0.50	11.34	0.18	1.58
13	54:7/56:6	62.34	0.06	14.61	0.12	0.85	91.40	0.52	14.01	0.19	1.39
14	54:8/56:7 (γ)	62.84	0.07	8.71	0.10	1.13	92.07	0.52	8.60	0.12	1.41
15	54:8/56:7	63.37	0.05	6.24	0.10	1.56	92.73	0.53	6.24	0.07	1.07
16	54:9 (γ)	63.88	0.04	2.88	0.06	2.21	93.40	0.54	2.84	0.11	3.73
17	54:9	64.43	0.04	1.18	0.09	7.40	94.10	0.56	1.24	0.08	6.27
18	54:10 (γ)	64.97	0.07	0.35	0.11	31.49	94.77	0.62	0.42	0.11	25.47

[a]From Lee Scientific. [b]Identifications are based on the spiked reference compounds and the mass spectrometric analysis. [c](γ) Denotes the triacylglycerols containing one γ-linolenic acid residue. [d]Five replicates. [e]Coefficient of variation.

exception to this trend was found in black currant seed oil, in which the proportion of the peak representing triacylglycerols 54:4 and 52:5 was nearly twice as high as the corresponding peak in the alpine currant seed oil.

The described method gives a new tool for achieving more information of the composition of triacylglycerols in the seed oils containing γ-linolenic acid. However, a method yielding more detailed separation is needed. Fractionation of triacylglycerols according to the number of acyl carbons before SFC on a 25% cyanopropyl–75% methylpolysiloxane stationary phase would be advantageous to enhance chromatographic resolution. The analysis and identification of co-eluting components having different ACNs could also be improved by combining SFC with MS. Nevertheless, chromatographic separation of triacylglycerols containing α- and γ-linolenic acid residues is important, because double-bond positional isomers of linolenic acid cannot be distinguished by MS without fractionation of the sample.

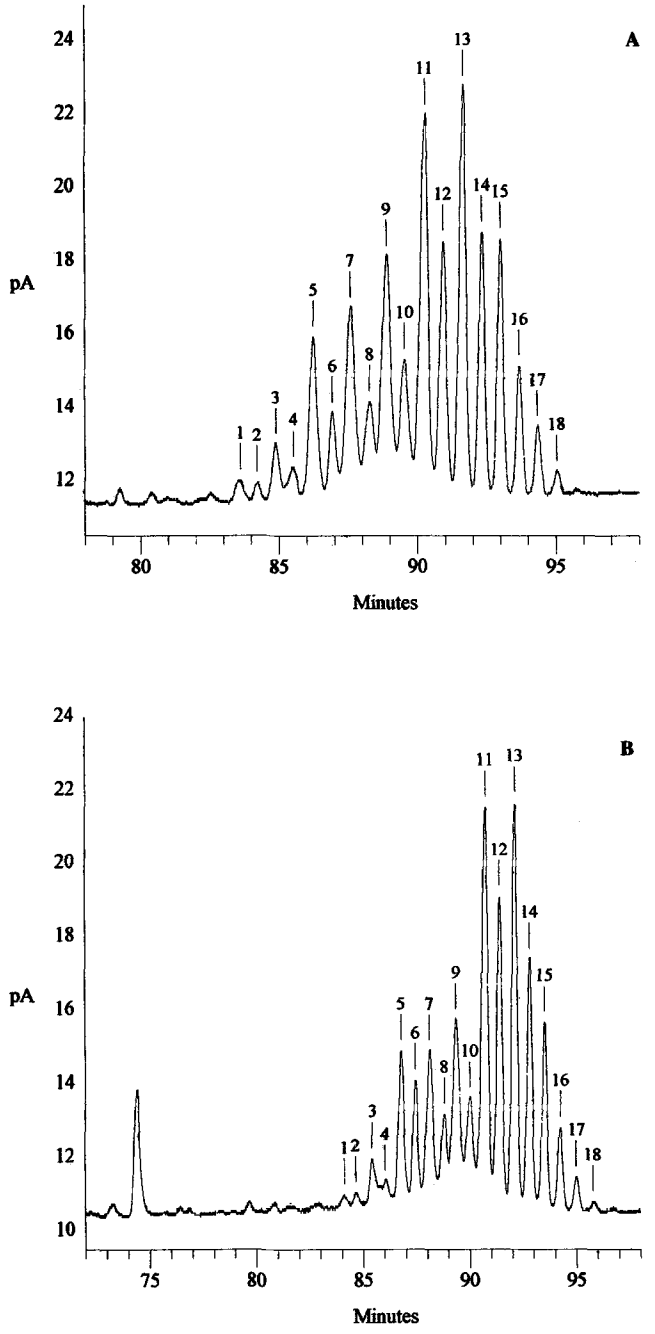


FIG. 5. Separation of triacylglycerols on an SB-Cyanopropyl-25 column (20 m × 50 μm i.d.; Lee Scientific, Dionex): (A) alpine currant seed oil, and (B) black currant seed oil. For peak identification see Table 2. See Figure 1 for company location.

ACKNOWLEDGMENTS

The authors thank Aromtech Oy for providing alpine currant and sea buckthorn seed oils, and Varsinais-Suomen Liitto for financial support of Pekka Manninen.

REFERENCES

1. Horrobin, D.F. (1992) Nutritional and Medical Importance of Gamma-Linolenic Acid, *Prog. Lipid Res. 31*, 163–194.
2. Gunstone, F.D. (1992) Gamma Linolenic Acid-Occurrence and Physical and Chemical Properties, *Prog. Lipid Res. 31*, 145–161.
3. Wolf, R.B., Kleiman, R., and England, R.E. (1983) New Sources of γ-Linolenic Acid, *J. Am. Oil Chem. Soc. 60*, 1858–1860.
4. Řezanka T., and Mareš, P. (1991) Determination of Plant Triacylglycerols Using Capillary Gas Chromatography, High-Performance Liquid Chromatography and Mass Spectrometry, *J. Chromatogr. 542*, 145–159.
5. Christie, W.W. (1993) Fractionation of the Triacylglycerols of Evening Primrose Oil by High-Performance Liquid Chromatography in Silver Ion Mode, *Fat Sci. Technol. 93*, 65–66.
6. Ratnayake, W.M.N., Matthews, D.G., and Ackman, R.G. (1989) Triacylglycerols of Evening Primrose (*Oenothera biennis*) Seed Oil, *J. Am. Oil Chem. Soc. 66*, 966–969.
7. Tsevegsüren, N., and Aitzetmüller, K. (1993) γ-Linolenic Acid in *Anemone* spp. Seed Lipids, *Lipids 28*, 841–846.
8. Perrin, J.-L., Prevot, A., Traitler, H., and Bracco, U. (1987) Analysis of Triglyceride Species of Blackcurrant Seed Oil by HPLC *via* a Laser Light Scattering Detector, *Rev. Fr. Corps Gras 34*, 221–222.
9. Aitzetmüller, K., and Grönheim, M. (1992), Separation of Highly Unsaturated Triacylglycerols by Reversed Phase HPLC with Short Wavelength UV Detection, *J. High Resolut. Chromatogr. 15*, 219–226.
10. Blomberg, L.G., Demirbüker, M., and Andersson, P.E. (1993) Argentation Supercritical Fluid Chromatography for Quantitative Analysis of Triacylglycerols, *J. Am. Oil Chem. Soc. 70*, 939–946.
11. Markides, K.E., Fields, S.M., and Lee, M.L. (1986) Capillary Supercritical Fluid Chromatography of Labile Carboxylic Acids, *J. Chromatogr. Sci. 24*, 254–257.
12. Christie, W.W. (1982) A Simple Procedure for Rapid Transmethylation of Glycerolipids and Cholesteryl Esters, *J. Lipid Res. 23*, 1072–1075.
13. Kallio, H., and Currie, G. (1993) Analysis of Low Erucic Acid Turnip Rapeseed Oil (*Brassica campestris*) by Negative Ion Chemical Ionization Tandem Mass Spectrometry. A Method Giving Information on the Fatty Acid Compositions *sn*-2 and *sn*-1/3 of Triacylglycerols, *Lipids 28*, 207–215.

[Received January 10, 1995, and in revised form April 10, 1995; Revision accepted April 11, 1995]

Lymphatic Fatty Acids from Rats Fed Human Milk and Formula Supplemented with Fish Oil

Richard M. Clark* and Li She

Department of Nutritional Sciences, University of Connecticut, Storrs, Connecticut 06269-4017

ABSTRACT: Absorption of long-chain polyunsaturated fatty acids from human milk and formula supplemented with fish oil was studied to determine if the distribution route into lymphatic triacylglycerol (TAG) and phospholipid (PL) varies with the dietary source. Rats were intraduodenally infused with human milk or formula containing graded amounts of fish oil (0, 0.5, or 1.0 g/100 mL), and the mesenteric lymph was collected. Arachidonic acid (20:4n-6) levels in lymphatic TAG and PL were highest from animals fed human milk. In the animals infused with formula containing fish oil, as the amount of eicosapentaenoic acid (EPA, 20:5n-3) infused increased, there was essentially an equal increase in EPA associated with both lymphatic TAG and PL. Animals intraduodenally infused with human milk or formula without fish oil had only minor levels (less than 1%) of EPA in the lymph. In the fish oil-treated animals, as the amount of docosahexaenoic acid (DHA, 22:6n-3) infused increased, there was a 16-fold increase in DHA associated with lymphatic TAG, but only a 3-fold increase in DHA associated with lymphatic PL. The highest level of DHA in rats infused with human milk was observed in lymphatic PL. Hence, fish oil can be added to formula as a source of long-chain polyunsaturated fatty acids, but the distribution of fatty acids into lymphatic TAG and PL is not the same as that observed with human milk.
Lipids 30, 673–676 (1995).

The rapidly growing neonate uses lipid from human milk for varied functions. Some of these functions include using it as an energy source, for the synthesis of membranes, and for the production of eicosanoids. To meet these varied needs, milk lipid contains a wide range of fatty acids, many of which are not found in infant formulas. The long-chain polyunsaturated fatty acids (LCPUFA) contained in human milk, but not in formulas, particularly arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), are of special interest because they are required by the neonate in the formation of neural and retinal tissue (1). Although AA and DHA can be synthesized from linoleic acid and α-linolenic acid, the neonate may not have the capacity to synthesize the amounts needed for normal development (2,3). Fish oils have been suggested as a possible source of LCPUFA for supplementation of infant formulas. Unfortunately, supplementation with fish oil has been reported to negatively affect infant growth and psychomotor development (4–6). It is thought that the much greater amounts of eicosapentaenoic acid (EPA, 20:5n-3) in fish oil, compared to human milk, may have adversely affected AA utilization by the infant (7). Because of the competition between n-6 and n-3 families of LCPUFA, further study is needed before appropriate recommendations can be made regarding LCPUFA supplementation of infant formulas.

The metabolic fate of a fatty acid is partially determined by its incorporation into different lipid classes of intestinal lipoproteins (8). The purpose of this study was to compare the distribution of fatty acids, particularly AA, EPA, and DHA, in lymphatic triacylglycerols (TAGs) and phospholipids (PLs) from rats fed human milk and infant formula containing corn oil supplemented with graded amounts of fish oil.

MATERIALS AND METHODS

Animals and surgical procedure. Male Holtzman albino rats obtained from Harlan Sprague Dawley (Indianapolis, IN) were used in this study. At the time of surgery, the rats weighed 300–375 g and were consuming a standard laboratory diet—Purina Rodent Chow 5001 (Purina Mills, St. Louis, MO)—and water *ad libitum.*

The major mesenteric lymph duct was cannulated as described by Warshaw (9). Surgery was performed under methoxyflurane–pentobarbital anesthesia. A right subcostal incision was made and a heparin-filled vinyl tube (medical grade, 0.50 mm i.d., 0.80 mm o.d.; Dural Plastics and Engineering, Dural, Australia) was threaded approximately 2 mm into the lymph duct. The cannula was secured using methylcyanoacrylate adhesive. A feeding tube was placed in the duodenum by passing Micro-renathane tubing (MRE 040; Braintree Scientific, Braintree, MA) in a caudal direction through a small gastric puncture and secured with methylcyanoacrylate adhesive. The abdominal wall was closed by suturing.

Immediately following surgery, the rats were placed in a warm, dark environment and allowed to recover for approximately 36 h. The rats had access to water and received a glu-

*To whom correspondence should be addressed at the Department of Nutritional Sciences, U-17, The University of Connecticut, Storrs, CT 06269-4017.

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; PL, phospholipid; TAG, triacylglycerol.

Copyright © 1995 by AOCS Press

cose/electrolyte solution (Pedialyte; Ross Laboratories, Columbus, OH) at 2.0 mL/h during the recovery period *via* the duodenal feeding tube.

Infusates. The liquid diets used in this study were human milk, infant formula, infant formula plus 0.5% fish oil (0.5 g/100 mL) and infant formula plus 1.0% fish oil (1.0 g/100 mL). The human milk was donated by a woman during her eighth month of lactation. The infant formula contained corn oil (Nutramigen; Mead Johnson, Evansville, IN). Fish oil concentrate (Consumer Value Stores, Melville Corp., Woonsocket, RI) was emulsified (Branson Sonifier, Model 185; Branson Sonic Power, Danbury, CT) in the formula at room temperature and a setting of "4" for 15 s, repeated four times.

Following their recovery, seven rats per diet were infused (2.0 mL/h) for 12 h through the feeding tube. After infusing the diet for 6 h, lymph was collected during the next 6 h of infusion. During the collection period lymph flow averaged 2.6 mL/h. The lymph was then stored at −70°C until analyzed.

Lipid analyses. Total lipid was extracted from aliquots of each diet and lymph collection by a modified Folch procedure (10). The nonpolar and polar lipid (total PLs) were separated on a column containing Unisil (Clarkson Chemical Co., Williamsport, PA). The TAG in the lymph was isolated from other neutral lipid on silica gel G thin-layer chromatographic plates developed with hexane/diethyl ether/glacial acetic acid (90:30:2, by vol).

Fatty acids associated with TAG and total PL were transmethylated in methanol/hexane (4:1, vol/vol) in the presence of acetyl chloride (11), and fatty acid methyl esters were separated by gas–liquid chromatography on a Supelcowax 10 fused silica capillary column (30 m, 0.53 mm i.d.; Supelco, Bellefonte, PA).

Statistical analyses. Statistical analyses were performed by one-way analysis of variance. Comparisons between individual diets were made using Student's *t*-test for unpaired variates.

TABLE 1
Fatty Acid Composition of Liquid Diets

	Formula[a]	Formula plus 0.5% fish oil[b]	Formula plus 1.0% fish oil[b]	Human milk
Total fatty acid (mg/mL)	23	28	33	32
Fatty acid (wt%)				
14:0	—[c]	2.0	3.1	6.3
16:0	11.1	12.0	12.3	24.2
16:1	—	2.5	3.7	2.2
18:0	2.5	2.4	2.3	9.5
18:1	24.2	22.0	20.0	31.5
18:2n-6	58.6	42.2	34.6	15.4
18:3n-3	2.4	1.9	1.7	0.8
20:4n-6	—	0.8	1.6	1.3
20:5n-3	—	5.0	8.2	0.8
22:6n-3	—	3.4	5.2	1.1

[a]Formula was Nutramigen (Mead Johnson, Evansville, IN) containing corn oil.
[b]Fish oil concentrate (Consumer Value Stores, Melville Corp., Woonsocket, RI): 0.5% fish oil was 0.5 g/100 mL formula. 1.0% fish oil was 1.0 g/100 mL formula.
[c]Not detected (less than 0.05 wt%).

RESULTS

The fatty acid compositions of the diets are shown in Table 1, and fatty acids in the lymph are shown in Table 2. The fatty acids in lymphatic TAG closely reflect the fatty acid composition of the diet, whereas PL was moderately affected.

The saturated fatty acid (14:0, 16:0, and 18:0) levels associated with lymphatic TAG in rats fed human milk were approximately twofold higher than those in the TAG from formula-fed rats. The saturated fatty acids were relatively constant in lymphatic PL and were not significantly influenced by diet. The monoenoic fatty acid (16:1 and 18:1) levels also were higher in the diet and lymph of the rats fed human milk compared to the animals fed formula. AA level was highest in the lymph of animals fed human milk.

TABLE 2
Effect of Diet on Fatty Acid Composition of Triacylglycerol and Phospholipid in Lymph

	Lymphatic fatty acids (wt%)					
	Total saturated	Total monoenoic	18:2n-6	20:4n-6	20:5n-3	22:6n-3
Lymphatic triacylglycerol						
Human milk	40.0 ± 2.0[a]	36.1 ± 2.3[a]	16.4 ± 1.5[a]	1.5 ± 0.5[a]	0.5 ± 0.2[a]	0.7 ± 0.4[a]
Formula	19.4 ± 2.2[b]	26.3 ± 4.3[b]	47.8 ± 5.8[b]	1.0 ± 0.8[a]	0.2 ± 0.2[b]	0.2 ± 0.2[b]
Formula + 0.5 FO[e]	21.4 ± 2.7[b]	25.4 ± 3.6[b]	38.8 ± 6.8[c]	1.1 ± 0.6[a]	2.6 ± 1.2[c]	2.2 ± 1.2[c]
Formula + 1.0 FO	20.9 ± 2.0[b]	25.1 ± 1.3[b]	36.0 ± 6.3[c]	0.9 ± 0.5[a]	4.6 ± 1.0[d]	3.2 ± 0.8[c]
Lymphatic phospholipid						
Human milk	42.0 ± 3.9[a]	16.2 ± 1.7[a]	28.3 ± 3.1[a]	8.2 ± 1.7[a]	0.8 ± 0.2[a]	1.8 ± 0.3[a]
Formula	39.4 ± 2.7[a]	10.9 ± 1.5[b]	38.4 ± 2.1[b]	6.7 ± 2.0[a,b]	0.3 ± 0.2[a]	0.6 ± 0.2[b]
Formula + 0.5 FO	39.9 ± 2.7[a]	11.2 ± 2.4[b,c]	30.9 ± 4.3[a]	6.2 ± 1.3[b]	2.5 ± 0.4[b]	1.1 ± 0.6[b,c]
Formula + 1.0 FO	39.2 ± 3.8[a]	13.5 ± 2.3[c]	28.8 ± 4.7[a]	5.5 ± 1.4[b]	4.2 ± 1.4[c]	1.6 ± 0.4[a,c]

[a]Values are means of lymph from seven rats ± SD. Means with different superscript letters (a,b,c,d) in the same column and lipid class are significantly different at $P < 0.05$.
[e]Fish oil.

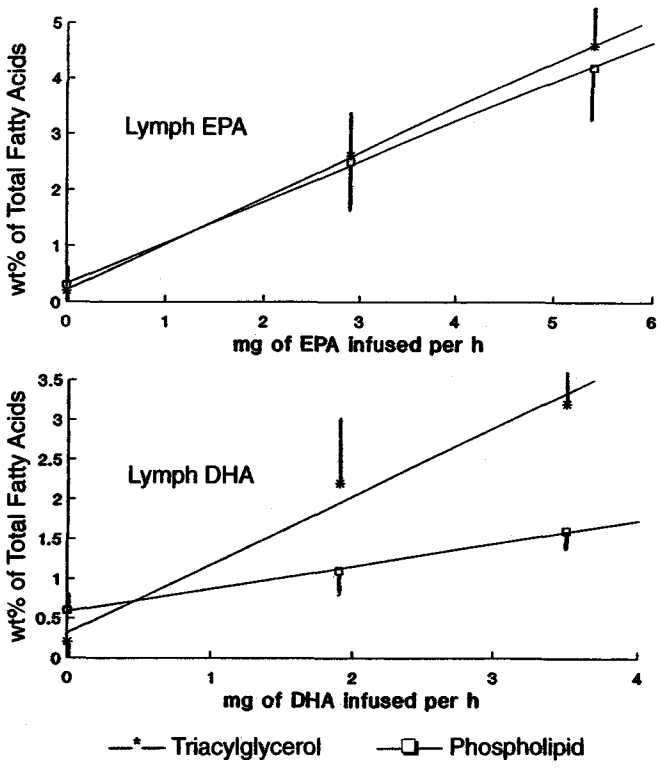


FIG. 1. Relationship between intraduodenal infusion of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and their distributions in lymphatic triacylglycerol and phospholipid. Each point is the average of seven observations ± SD.

The major difference among diets was the EPA associated with feeding fish oil. EPA was less than 1% of the fatty acids in the lymph from rats fed human milk and formula without fish oil and more than 4% in the lymph from rats fed formula supplemented with 1% fish oil. The proportion of EPA in the lymph was dose-dependent on the fish oil infused. In Figure 1, the proportions of EPA in lymphatic TAG and PL from rats fed formula with graded levels of fish oil are shown. As the amount of EPA infused increased, there was essentially an equal increase in EPA associated with both lymphatic TAG and PL.

DHA increased in the lymph with the amount of fish oil infused. However, as shown in Figure 1, DHA did not appear to be incorporated equally into TAG and PL in the lymph. As the amount of DHA intraduodenally infused increased, there was a 16-fold increase in DHA associated with lymphatic TAG, but only a 3-fold increase in DHA associated with lymphatic PL. In the human milk-fed animals, the lymphatic PL had a higher proportion of DHA than did lymphatic TAG. DHA was absorbed from both human milk and formula supplemented with fish oil, but its distribution route into lymphatic TAG and PL varied with the dietary source.

DISCUSSION

It has been proposed that LCPUFA be added to infant formulas, particularly those designed for low-birth-weight infants. Fish oil has been suggested as one source of LCPUFA. However, there are concerns about the use of fish oil, which include the bioavailability of LCPUFA from fish oil, and the EPA competitive inhibition of AA metabolism.

Reports on the bioavailability of LCPUFA from fish oils have not been consistent. Decreased absorption of EPA and DHA from fish oil compared to absorption of fatty acids from other oils has been reported in rats (12) and in humans (13), and other experiments have suggested more complete absorption (14–16). These differences may be due to experimental approach or previous diet. Rats previously fed fish oil had a greater apparent absorption of EPA and DHA from a test dose of fish oil than those fed corn oil (16). The well established resistance of fish oil to hydrolysis by pancreatic lipase is probably not physiologically important and not a factor affecting bioavailability (17).

In the present study, the proportion of EPA and DHA in the lymph from animals fed fish oil is only about 50% of that observed in the original diet (Table 1 vs. Table 2). This is in agreement with a similar study, which observed 55% less EPA and DHA as a proportion of total fatty acids in the lymph compared to the fatty acid composition of the fish oil fed (15). Although bioavailability may be a factor in the lower proportions of LCPUFA in the lymph, the present study was not designed to measure this. The actual quantities of fatty acids were not measured, and the length of time lymph was collected would not have been sufficient to recover all of the LCPUFA infused (16).

Figure 1 shows that DHA from fish oil was not incorporated equally into lymphatic TAG and PL. This difference in distribution of DHA in lymphatic PL compared to TAG may be explained by the structure of TAG in the fish oil supplement, positional specificity of pancreatic lipase, and the pathways for lipid synthesis in the enterocyte. Based on recent analyses of TAG from different fish oil supplements, DHA has been reported enriched in the *sn*-2 (15) or *sn*-2,3 position (13,18), whereas EPA was randomly distributed or enriched in the *sn*-3 position (13,15,18). Pancreatic lipase is known to hydrolyze fatty acids from the primary (*sn*-1,3) positions of menhaden oil (19). As a result of pancreatic lipase specificity, the *sn*-2 monoacylglycerol in the intestinal lumen of rats fed fish oil is enriched with DHA compared to the free fatty acids liberated from the glycerol (19). These products of lipid digestion are absorbed into the enterocyte where *sn*-2 monoacylglycerol enriched with DHA and two molecules of fatty acyl CoA form lymphatic TAG *via* the *sn*-2 monoacylglycerol pathway. The PL in lymph, mainly phosphatidylcholine, is synthesized in the enterocyte by acylation of lysophosphatidylcholine. Therefore, DHA in lymphatic TAG originates from the DHA-enriched *sn*-2 monoacylglycerol, while DHA associated with lymphatic PL originates from the less enriched DHA free fatty acid pool. Because EPA is not enriched in the *sn*-2 position of fish oil (13,15,18), it appears equally incorporated in lymphatic TAG and PL.

The second concern with adding fish oil to formulas was that the EPA in fish oil may negatively affect AA status. AA

in lymphatic PL was greatest in animals fed human milk and significantly ($P < 0.05$) less in animals fed formula with 1% fish oil. A similar trend ($P < 0.10$) was observed with lymphatic TAG (Table 2). The EPA/AA ratio also should be considered as it is a better index of AA status than AA alone (20). The EPA/AA ratio was 15-fold higher in lymphatic TAG and 8-fold higher in lymphatic PL from animals fed 1% fish oil formula compared to those fed human milk. This increase in EPA/AA ratio could suppress AA metabolism and potentially cause undesirable side effects such as increased risk of bleeding, altered immune function, and decreased neonatal growth and development (4–7).

In summary, fish oil can be added to formula to increase total DHA in lymph comparable to amounts seen with human milk. However, the distribution of DHA between lipid classes in the lymph is different. Lymphatic PL is most enriched with DHA in animals fed human milk, while in fish oil-supplemented animals lymphatic TAG accumulates DHA to a greater degree than PL. This difference may affect the metabolic fate of DHA as the fatty acids bound to lymphatic TAG are metabolized differently than those bound to the PL fraction of chylomicrons. Fish oil supplementation also increases the EPA/AA ratio 8- to 16-fold in lymphatic PL and TAG. Based on the EPA/AA ratio in lymph, adding fish oil to formula would have a negative effect on AA status.

ACKNOWLEDGMENTS

Supported in part by Federal Funds available through provisions of the Hatch Act. This work constitutes Scientific Contribution No. 1560 Storrs Agricultural Experiment Station, The University of Connecticut, Storrs, CT.

REFERENCES

1. Clandinin, M.T., Chappell, J.E., Leong, S., Heim, T., Swyer, P.R., and Chance, G.W. (1980) Intrauterine Fatty Acid Accretion Rates in Human Brain: Implications for Fatty Acid Requirements, *Early Human Dev. 4*, 121–130.
2. Uauy, R.D., Birch, D.G., Birch, E.E., Tyson, J.E., and Hoffman, D.R. (1990) Effect of Dietary Omega-3 Fatty Acids on Retinal Function of Very-Low-Birth-Weight Neonates, *Pediatr. Res. 28*, 485–492.
3. Clark, K.J., Makrides, M., Neumann, M.A., and Gibson, R.A. (1992) Determination of the Optimal Ratio of Linoleic Acid to Alpha-Linolenic Acid in Infant Formulas, *J. Pediatr. 120*, S151–S158.
4. Carlson, S.E., Cooke, R.J., Rhodes, P.G., Peeples, J.M., Werkman, S.H., and Tolley, E.A. (1991) Long-Term Feeding of Formulas High in Linolenic Acid and Marine Oil to Very Low Birth Weight Infants: Phospholipid Fatty Acids, *Pediatr. Res. 30*, 404–412.
5. Carlson, S.E., Cooke, R.J., Werkman, S.H., and Tolley, E.A. (1992) First Year Growth of Preterm Infants Fed Standard Compared to Marine Oil n-3 Supplemented Formula, *Lipids 27*, 901–907.
6. Carlson, S.E., Werkman, S.H., Peeples, J.M., Cooke, R.J., Tolley, E.A., and Wilson, W.M. (1993) Growth and Development of Very Low-Birth Weight Infants in Relation to n-3 and n-6 Essential Fatty Acid Status, in *Essential Fatty Acids and Eicosanoids: Invited Papers from the Third International Congress* (Sinclair, A., and Gibson, R., eds.) pp. 192–196, AOCS Press, Champaign.
7. Gibson, R.A. (1993) What is the Best Fatty Acid Composition for the Fats of Infant Formulas, in *Essential Fatty Acids and Eicosanoids: Invited Papers from the Third International Congress* (Sinclair, A., and Gibson, R., eds.) pp. 210–213, AOCS Press, Champaign.
8. Nilsson, A., Hjelte, L., and Strandvik, B. (1992) Incorporation of Dietary ^{14}C Arachidonic Acid and ^{3}H Eicosapentaenoic Acid Into Tissue Lipids During Absorption of a Fish Oil Emulsion, *J. Lipid Res. 33*, 1295–1305.
9. Warshaw, A.L. (1972) A Simplified Method of Cannulating the Intestinal Lymphatic of the Rat, *Gut 13*, 66–67.
10. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
11. Lepage, G., and Roy, C. (1986) Direct Transesterification of All Classes of Lipids in a One-Step Reaction, *J. Lipid Res. 27*, 114–120.
12. Chen, I.S., Hotta, S.S., Ikeda, I., Cassidy, M.M, Sheppard, A.J., and Vahouny, G.V. (1987) Digestion, Absorption and Effects on Cholesterol Absorption of Menhaden Oil, Fish Oil Concentrate and Corn Oil By Rats, *J. Nutr. 117*, 1676–1680.
13. Lawson, L.D., and Hughes, B.G. (1988) Human Absorption of Fish Oil Fatty Acids as Triacylglycerols, Free Acids, or Ethyl Esters, *Biochem. Biophys. Res. Comm. 152*, 328–335.
14. Chen, I.S., Subramaniam, S., Cassidy, M.M., Sheppard, A.J., and Vahouny, G.V. (1985) Intestinal Absorption and Lipoprotein Transport of (Omega-3) Eicosapentaenoic Acid, *J. Nutr. 115*, 219–225.
15. Chernenko, G.A., Barrowman, J.A., Kean, K.T., Herzberg, G.R., and Keough, K.M.W. (1989) Intestinal Absorption and Lymphatic Transport of Fish Oil (MaxEPA) in the Rat, *Biochem. Biophys. Acta 1004*, 95–102.
16. Herzberg, G.R., Chernenko, G.A., Barrowman, J.A., Kean, K.T., and Keough, K.M.W. (1992) Intestinal Absorption of Fish Oil in Rats Previously Adapted to Diets Containing Fish Oil or Corn Oil, *Biochim. Biophys. Acta 1124*, 190–194.
17. Yang, L-Y., Kuksis, A., and Myher, J. (1990) Lipolysis of Menhaden Oil Triacylglycerols and the Corresponding Fatty Acid Alkyl Esters by Pancreatic Lipase In Vitro: a Reexamination, *J. Lipid Res. 31*, 137–148.
18. Leray, C., Raclot, T., and Groscolas, R. (1993) Positional Distribution of n-3 Fatty Acids in Triacylglycerols from Rat Adipose Tissue During Fish Oil Feeding, *Lipids 28*, 279–284.
19. Yang, L-Y., Kuksis, A., and Myher, J. (1989) Lumenal Hydrolysis of Menhaden and Rapeseed Oils and Their Fatty Acid Methyl and Ethyl Esters in the Rat, *Biochem. Cell Biol. 67*, 192–204.
20. Boudreau, M.D., Chanmugam, P.S., Hart, S.B., Lee, S.H., and Hwang, D.H. (1991) Lack of Dose Response by Dietary n-3 Fatty Acids at a Constant Ratio of n-3 to n-6 Fatty Acids in Suppressing Eicosanoid Biosynthesis from Arachidonic Acid, *Am. J. Clin. Nutr. 54*, 111–117.

[Received July 13, 1994, and in revised form March 6, 1995; Revision accepted April 7, 1995]

Comparison of the Fatty Acids of the Tunicate *Botryllus schlosseri* from the Black Sea with Two Associated Bacterial Strains

Néstor M. Carballeira[a,*], Fathi Shalabi[a], Kamen Stefanov[b], Krassimir Dimitrov[b], Simeon Popov[b], Athanas Kujumgiev[c] and Stoitze Andreev[d]

[a]Department of Chemistry, University of Puerto Rico, San Juan, Puerto Rico 00931-3346, [b]Institute of Organic Chemistry with Center of Phytochemistry, [c]Institute of Microbiology, and [d]Museum of Natural History, Bulgarian Academy of Sciences, Sofia 1000, Bulgaria

ABSTRACT: The fatty acid composition of the tunicate *Botryllus schlosseri* and of two bacterial strains found within the tunicate, namely *Vibrio parahaemolyticus* and of an associated but previously unreported gram positive cocci were studied. The polyunsaturated fatty acids 6,9,12-octadecatrienoic acid, 5,8,11,14,17-eicosapentaenoic acid, and 4,7,10,13,16,19-docosahexaenoic acid were particularly abundant in *B. schlosseri* and were not detected in the two bacterial strains found in the tunicate. The *iso/anteiso* pair, 13-methyltetradecanoic acid and 12-methyltetradecanoic acid, were the principal fatty acids in the gram positive cocci, and the 9- and 11-hexadecenoic acids were particularly abundant in *V. parahaemolyticus*. The diunsaturated fatty acid 9,12-octadecadienoic acid was also shown to be present in *V. parahaemolyticus*. The fatty acid composition of a third bacterial strain, characterized as either a *Pseudomonas* or an *Alteromonas species*, and shown to be present only in the sea water from the Black Sea and not in *B. schlosseri*, is also reported. This is the first investigation on fatty acids from Black Sea bacteria.

Lipids 30, 677–679 (1995).

There are only a few investigations on the lipid composition of tunicates. Probably, the most comprehensive study has been reported by Kostetsky and collaborators (1,2), where the fatty acid composition of phosphatidylethanolamines and phosphatidylcholines from *Botryllus tuberatus* and *Botryloides eligulatum* were reported. The main fatty acids in these phospholipids were identified as 16:0, 18:2ω6, 20:4ω6, 20:5ω3, and 22:6ω3. However, some of the double-bond positions in the monoenoic fatty acids from these tunicates were not determined. Moreover, there is no known report on the bacterial composition of tunicates. A careful study of the fatty acid composition of the different bacteria present in the tunicate could reveal which fatty acids originate from the bacteria and which arise from tunicate cells. For example, it is known that cultures of *Shewanella putrefaciens* strain ACAM 342, grown aerobically and anaerobically at 15 and 25°C, produce the polyunsaturated fatty acids 18:2ω3, 18:3ω3, and 20:5ω3 (3). Some of the latter fatty acids, such as 20:5ω3, are also known to be present in tunicates. *Shewanella putrefaciens* is a common facultative anaerobe from the Black Sea, and reduces thiosulfate, sulfite, and elemental sulfur to sulfide (4).

In the present study, we report the fatty acid composition of the colonial tunicate *Botryllus schlosseri* and two associated bacteria from the Black Sea. To the best of our knowledge, there are no reports on the fatty acid composition of *B. schlosseri*. It was also of interest to determine if a bacteria such as *S. putrefaciens* was present in *B. schlosseri*. Are the polyunsaturated fatty acids in tunicates produced by bacterial symbionts? Which fatty acids are produced by bacterial associates and which are produced by the tunicate? These are the questions that we answer in the present study.

MATERIALS AND METHODS

Sample. The tunicate was collected September 10, 1993, near Ravda (South Black Sea coast of Bulgaria) at 20 m from the beach and at a depth of 2–3 m. Water samples were also collected from the same place.

Bacterial isolation. The bacteria were obtained from both *B. schlosseri* and sea water from the collection sites. *Botryllus schlosseri* was cut into small pieces and ground in a porcelain mortar. The homogenates obtained were diluted in standard nutrient broth with a final salt concentration of 1.6% (equal to the concentration of salts in the Black Sea). The following dilutions were used: 10^{-1} (0.1 mL water or homogenate + 0.9 mL broth), 10^{-2}, 10^{-3}, and 10^{-4}. From every dilution, 0.1 mL were taken and inoculated into two Petri dishes containing standard nutrient agar with a final concentration of NaCl 1.6%. After incubation at 37°C for 24 h, we

* To whom correspondence should be addressed at the Department of Chemistry, University of Puerto Rico, P.O. Box 23346, San Juan, Puerto Rico 00931-3346.

Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

Copyright © 1995 by AOCS Press

established the morphology of the material obtained. We transferred each strain to a separate flask and cultivated them in the standard nutrient broth with aeration at 37°C for 24 h. The bacterial biomass obtained from every strain was separated by centrifugation of the cultured liquid (6000 r.p.m. for 20 min) and the lipids were extracted as described by Christie *et al.* (5).

Bacterial identification. Characterization of the bacteria was done by standard biochemical tests modified for marine bacteria (6–8). The API 20E system (Analytab Products, Plainview, NY) was also used for the marine isolates. The three samples were identified as *Pseudomonas–Alteromonas* (OB-1), gram positive cocci (OB-2), and *Vibrio parahaemolyticus* (OB-3).

Fatty acid isolation. The lipids from *B. schlosseri* and from the three bacterial strains were extracted with chloroform/methanol (2:1, vol/vol) and saponified, the nonsaponifiables were removed, and the acids were methylated with methanol containing 1% sulfuric acid (5).

Instrumentation. Gas chromatography (GC) spectra were recorded on a Hewlett-Packard (Palo Alto, CA) 5890A gas chromatograph equipped with a flame-ionization detector and a nonpolar fused silica capillary column (30 m × 0.25 mm i.d.) containing DB-1 (carrier gas He). GC/mass spectrometry (GC/MS) data was collected at 70 eV in a Hewlett-Packard 5972A MS ChemStation equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS), similar, but crosslinked with 5% phenyl methylpolysiloxane. The temperature program for both GC and GC/MS analyses was as follows: 130°C for 2 min, then increased at 3°C/min to 270°C and maintained for 40 min.

Derivatives. Hydrogenations were carried out in 10 mL of absolute methanol and catalytic amounts of platinum oxide (PtO_2). The double-bond positions of the mono- and dienoic fatty acids were determined by preparing the corresponding dimethyl disulfide derivatives by dissolving the esters (2 mg) in dimethyldisulfide (0.2 mL), adding a solution (0.05 mL) of I_2 in Et_2O (60 mg/mL), and heating the solution at 50°C for 24 h, followed by the standard work-up (9,10).

RESULTS AND DISCUSSION

In the present study, the total fatty acids from the tunicate *B. schlosseri* were analyzed together with three bacterial strains from the sea water, two of which were found in the tunicate. The fatty acid profile of *B. schlosseri* is presented in Table 1. Saturated normal-chain fatty acids accounted for almost 40% of the total fatty acid composition of *B. schlosseri*, hexadecanoic acid (21%) and octadecanoic acid (6%) being the most prominent fatty acids in the mixture. Saturated branched-chain fatty acids accounted for only 4.3% of the total composition, the *iso/anteiso* 15:0 and 17:0 acids being the most abundant. As expected, the polyunsaturated fatty acids 20:5ω3 and 22:6ω3 were the main fatty acids in the tunicate, accounting for 26% of the total composition. The common marine fatty acids 20:4ω3 and 22:5ω3 were not found in *B. schlosseri*. Similar results were observed by Kostetsky and co-workers in *B. tuberatus* and in *Botrylloides eligulatum* (1,2). The most important dienoic fatty acids characterized in *B. schlosseri* were 9,12-octadecadienoic acid (18:2), 5,11-

TABLE 1
The Fatty Acid Composition (wt% of the total) from the Tunicate *Botryllus schlosseri* and the Three Associated Bacterial Strains

Fatty acid	Abundance (wt %)			
	B. schlosseri	OB-1[a]	OB-2[b]	OB-3[c]
Dodecanoic (12:0)	0.1	—	0.1	0.1
Tridecanoic (13:0)	0.2	0.2	0.8	0.1
7-Tetradecenoic (14:1)	—	—	—	0.2
11-Methyltridecanoic (*ai*-14:0)	0.2	0.3	1.1	0.1
Tetradecanoic (14:0)	3.8	0.4	1.0	2.1
13-Methyltetradecanoic (*i*-15:0)	1.0	23.5	52.0	1.4
12-Methyltetradecanoic (*ai*-15:0)	0.6	—	22.0	0.1
Pentadecanoic (15:0)	1.5	0.6	1.0	0.9
14-Methylpentadecanoic (*i*-16:0)	0.8	0.2	0.5	0.3
9-Hexadecenoic (16:1)	3.1	17.9	4.7	31.8
11-Hexadecenoic (16:1)	1.3	—	—	5.6
Hexadecanoic (16:0)	20.8	22.3	6.2	29.3
15-Methylhexadecanoic (*i*-17:0)	0.9	8.7	2.5	2.1
14-Methylhexadecanoic (*ai*-17:0)	0.8	—	—	0.5
9-Heptadecenoic (17:1)	—	1.1	0.8	0.2
10-Heptadecenoic (17:1)	—	—	—	0.2
11-Heptadecenoic (17:1)	—	0.3	—	0.2
Heptadecanoic (17:0)	4.8	3.6	0.7	1.3
6,9,12-Octadecatrienoic (18:3n-6)	2.1	—	—	—
9,12-Octadecadienoic (18:2)	2.3	—	0.1	1.7
9-Octadecenoic (18:1)	6.3	15.1	0.8	—
11-Octadecenoic (18:1)	3.6	3.6	0.6	10.6
13-Octadecenoic (18:1)	—	—	—	3.2
Octadecanoic (18:0)	6.1	1.3	0.7	3.9
17-Methyloctadecanoic (*i*-19:0)	—	0.1	—	0.4
11-Nonadecenoic (19:1)	—	0.2	—	0.1
Nonadecanoic (19:0)	0.8	0.1	0.1	0.1
5,8,11,14-Eicosatetraenoic (20:4n-6)	1.6	—	—	—
5,8,11,14,17-Eicosapentaenoic (20:5n-3)	10.5	—	—	—
5,11-Eicosadienoic (20:2)	1.0	—	—	—
11-Eicosenoic (20:1)	1.7	—	—	—
13-Eicosenoic (20:1)	1.9	—	—	—
Eicosanoic (20:0)	1.1	—	0.8	0.3
4,7,10,13,16-Docosapentaenoic(22:5n-6)	1.8	—	—	—
4,7,10,13,16,19-Docosahexaenoic(22:6n-3)	15.5	—	—	—
7,15-Docosadienoic (22:2)	2.1	—	—	—
Tetracosanoic (24:0)	0.3	—	0.2	0.1

[a]The OB-1 bacteria was identified as a *Pseudomonas–Alteromonas*. It was only detected in the sea water and not in the tunicate.
[b]The OB-2 bacteria was identified as a gram positive cocci. It was detected in both the tunicate and in the sea water.
[c]The OB-3 bacteria was identified as *Vibrio parahaemolyticus*. It was also detected in both the tunicate and in the sea water.

eicosadienoic acid (20:2), and 7,15-docosadienoic acid (22:2). The double-bond positions in these dienoic fatty acids were determined by GC/MS analysis on the corresponding dimethyl disulfide derivatives prepared and analyzed as previously described (10).

Two bacterial strains, designated as OB–2 and OB–3, were isolated from *B. schlosseri* and a third, OB–1, only detected in the sea water where the tunicate was collected, was also isolated. The strain OB-2, a new bacteria, could only be identified as a gram positive cocci since the biochemical characteristics did not match those of any known bacteria. The fatty acid profile of OB-2 is also presented in Table 1. The most important fatty acids in OB-2 were the 13-methyltetradecanoic (*i*-15:0) and the 12-methyltetradecanoic (*ai*-15:0), which accounted for 74% of the total fatty acid composition of this gram positive cocci. These fatty acids were characterized by GC/MS, GC retention times, and GC co-injection with authentic standards. Therefore, OB-2 seems to be responsible for most of the *isolanteiso* 15:0 fatty acids found in the tunicate.

The second bacterial strain present in the tunicate, namely OB-3, was characterized as *V. parahaemolyticus* as described in the Materials and Methods section. *Vibrio parahaemolyticus* showed the more common even-chain fatty acids. The key fatty acids in *V. parahaemolyticus* were 9-hexadecenoic acid (16:1), hexadecanoic acid (16:0), 11-octadecenoic acid (18:1), and octadecanoic acid (18:0), which together accounted for 76% of the total fatty acids of *V. parahaemolyticus*. Therefore, *V. parahaemolyticus* seems to be the major supplier of even-chain ω7 fatty acids for *B. schlosseri*. Moreover, *V. parahaemolyticus* was the only bacterial strain tested in this study to produce 11-hexadecenoic acid (16:1) and 14-methylhexadecanoic acid (*ai*-17:0). It is also of interest to note that the 9,12-octadecadienoic acid of bacterial origin was present in both OB-2 and OB-3, and these bacteria are likely to be the suppliers of this fatty acid for *B. schlosseri*. Are bacteria the source of the 9,12-octadecadienoic acid in marine invertebrates? More research has to be done on other marine invertebrates to answer this question, but this seems to be a very likely possibility.

A third bacterial strain, namely OB-1, could only be characterized as a *Pseudomonas–Alteromonas*. The biochemistry did not match that of any known bacteria. This strain was only found in the sea water where the tunicate was collected and not in *B. schlosseri*. Therefore, this seems to be a bacterium typical for the collection period and place in the Black Sea. Aerobic marine bacteria of the genera *Alteromonas*, *Pseudomonas*, and *Shewanella* have often been confused, due to phenotypic similarities (11). Therefore, because these marine bacteria share similar physiological and biochemical characteristics, more comprehensive tests, such as rRNA–DNA hybridization studies, must be carried out in order to fully characterize this bacterium (11). It is very likely that OB-1 will be closer to the *Alteromonas* as many *Pseudomonas* species have been found to contain cyclopropane and 2-hydroxy fatty acids (12). Such acids were not found in OB-1. The most abundant fatty acids in OB-1 were 13-methyltetradecanoic acid (*i*-15:0), 9-hexadecenoic acid (16:1), hexadecanoic acid (16:0), and 9-octadecenoic acid (18:1).

The results presented in this work are of interest because they clearly define which fatty acids in *B. schlosseri* are of bacterial origin and which are from the tunicate itself. From our results, it can be concluded that the more highly polyunsaturated fatty acids in *B. schlosseri*, namely 18:3ω6, 20:4ω6, 20:5ω3, 22:6ω3, and 22:5ω6, are not of bacterial origin. Although true symbiosis is difficult to assess in marine organisms, it is very likely that the bacteria found in *B. schlosseri*, the source of most of the short-chain saturated and monounsaturated fatty acids in the tunicate, are in symbiosis with the tunicate. Work is in progress toward the elucidation of the origin of unusual fatty acids in marine invertebrates.

ACKNOWLEDGMENTS

We thank Dr. Thomas Tosteson and Carmen Tosteson from the Department of Marine Sciences, University of Puerto Rico, Mayagüez campus, and Dr. Gary Toranzos, Department of Biology, University of Puerto Rico, Río Piedras campus, for the classification of the bacteria. We thank the Puerto Rico (EPSCoR) Tropical Marine Biotechnology Center for financial support as well as the National Science Foundation MRCE program. This work was also supported by the National Institutes of Health (NIH-MBRS Program) under Grant No. S06 GM08102-20 and by the National Foundation of Scientific Research of Bulgaria under contract X-443.

REFERENCES

1. Kostetsky, E.Y., and Naumenko, N.V. (1984) *Khim. Prirod. Soed. (USSR) 1*, 24–29.
2. Kostetsky, E.Y., Naumenko, N.V., and Gerasimenko, N.I. (1983) *Biologiya Morya (USSR) 2*, 51–56.
3. Nichols, D.S., Nichols, P.D., and McMeekin, T.A. (1992) *FEMS Microbiol. Lett. 98*, 117–122.
4. Perry, K.A., Kostka, J.E., Luther III, G.W., and Nealson, K.H. (1993) *Science 259*, 801–803.
5. Christie, W.W., Brechany, E.Y., Stefanov, K., and Popov, S. (1992) *Lipids 27*, 640–644.
6. King, N.R., and Holt, J.G. (1984) *Bergey's Manual of Systematic Bacteriology*, Vol. 1, The Williams and Wilkins Co., Baltimore.
7. Colwell, R.R. (1984) *Vibrios in the Environment*, John Wiley & Sons, New York.
8. Oliver, J.D. (1982) *Taxonomic Scheme for the Identification of Marine Bacteria 29*, 795–798.
9. Francis, G., and Veland, K. (1981) *J. Chromatogr. 219*, 379–384.
10. Carballeira, N., Shalabi, F., and Cruz, C. (1994) *Tetrahedron Lett. 35*, 5575–5578.
11. Akagawa-Matsushita, M., Itoh, T., Katayama, Y., Kuraishi, H., Yamasato, K. (1992) *J. Gen. Microbiol. 138*, 2275–2281.
12. Galbraith, L., and Wilkinson, S. (1991) *J. Gen. Microbiol. 137*, 197–202.

[Received December 21, 1994, and in revised form April 10, 1995; Revision accepted April 17, 1995]

ERRATUM

Figures 1 and 2 were transposed in the paper by T. Wieder *et al.*, The Effect of Two Synthetic Phospholipids on Cell Proliferation and Phosphatidylcholine Biosynthesis in Madin-Darby Canine Kidney Cells, *Lipids 30,* 389–393 (May 1995). The captions are correct.

ANNOUNCEMENTS

CALENDAR

August 1995
36th International Conference on the Biochemistry of Lipids, Aug. 8–11, Georgetown University, Washington, D.C., USA. Information: Ms. Hattie Johnson, Georgetown University, Office of Continuing Professional Education, 2233 Wisconsin Ave., NW, Suite 333, Washington, DC 20007-2197 USA (Phone: 202-687-8735; Toll-Free: 800-382-3613; Fax: 202-687-3019).

September 1995
5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October 1995
21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

American College of Nutrition 36th Annual Meeting, Oct. 13–15, 1995, Omni Shoreham Hotel, Washington, D.C. Information: American College of Nutrition, c/o Hospital for Joint Disease, 301 E. 17th St., New York, NY 10003 (Fax: 212-777-1103).

Lipidforum Seminar: Lipids in Animal Nutrition, Oct. 25–26, 1995, Research Centre Foulum, Tjele (Viborg), Denmark. Information: G. Lambertsen, Lipidforum, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-32 57 24).

November 1995
XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

February 1996
Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

July 1996
European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

July–August 1997
Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Gas Chromatography/Tandem Mass Spectrometry as an Analytical Tool for the Identification of Fatty Acids

F. Couderc*

Institut de Biologie Cellulaire et Génétique du CNRS, 31062 Toulouse Cedex, France

ABSTRACT: Structures of fatty acids present at very low quantities in mycobacteria are difficult to determine. A commonly used strategy is to introduce heteroatoms into functional groups by chemical means before subjecting them to gas chromatography/tandem mass spectrometry (GC/MS/MS) analysis. Routinely used methods give very low abundance diagnostic ions leading to ambiguities in structural conclusions. GC/MS/MS associated with electron capture ionization of pentafluorobenzyl esters was used to study very complex mixtures of fatty acids from *Mycobacterium fallax* and *M. aurum*. The charge–remote fragmentation of fatty acid carboxylate anions was used for structure determination at the nanogram level of a large number of unsaturated, branched, and cyclopropane-containing fatty acids. Some of them have not been observed previously in these Mycobacteria. On the basis of these studies, biosynthetic pathways of unsaturated, branched, and cyclopropane-containing fatty acid are proposed.

Lipids 30, 691-699 (1995).

Mycobacteria synthesize considerable quantities of a wide variety of fatty acids (1). In *Mycobacterium phlei*, for example, more than 40 different fatty acids, from C_{12} to C_{36}, have been detected by gas chromatography/mass spectrometry (GC/MS) (2). Although it is not difficult to characterize the saturated fatty acids by GC/MS by electron ionization (EI), the location of double bonds, cyclopropane rings, or branched points is more difficult. On EI, it is possible for double bonds and cyclopropane rings to migrate, making their spectra difficult to interpret (3,4).

A widely used strategy is to introduce heteroatoms into these functional groups by chemical means before subjecting them to GC/MS analysis (5–9). The fragmentation pattern is markedly influenced by the presence of these heteroatoms, and the position of the ring structures and double bonds can be deduced from the characteristic EI spectra. The various methods, however, give very low abundance diagnostic ions, leading to ambiguities in structural conclusions (7,9).

It was shown that using collision activated dissociation/(CAD) mass analyzed ion kinetic energy spectra (MIKES) of aliphatic ions containing a strongly localized charge provides an easy and accurate method for analysis of fatty acids (charge-remote fragmentation). In particular, monounsaturated (10), α,β-unsaturated (11), polyunsaturated (12,13), methyl branched (14), methylene branched (15,16), cyclopropyl (17), cyclopentyl, and cyclohexyl (18) rings are easily detected and located.

Some years ago, we developed a new method that allows straightforward structural determination of fatty acids coming from very complex mixtures (19–21). Direct analysis of $(M - H)^-$ of fatty acids desorbed by fast atom bombardment and collisionally activated negative ion tandem mass spectrometry (MS/MS) was unsuccessful because many isomeric ions were present (19). Thus a chromatographic separation (as GC) was necessary. As we proposed earlier (19–21), the present paper describes a GC/MS/MS investigation of the fatty acids mixture from *M. fallax* and *M. aurum* by using charge–remote fragmentation process of collisionally activated carboxylate anions generated in the gas phase by electron capture of fatty acid pentafluorobenzyl esters (PFBE).

MATERIALS AND METHODS

Bacteria and growth conditions. Strains were obtained from the Collection of the Institut Pasteur (CIP) (Paris, France) and from the American Type Culture Collection (ATCC) (Rockville, MD). The CIP 8139 strain of *M. fallax* and ATCC 10437 of *M. aurum* were grown under agitation in 2-L Erlenmeyer flasks containing 500 mL of Middelbrook medium 7H9 (Difco, Detroit, MI), supplemented with 0.5% casitone (Difco) and 1% glucose (22). *Mycobacterium fallax* was cultivated at 30°C and *M. aurum* at 37°C. The bacteria were collected by centrifugation at the end of the exponential growth period. *Mycobacterium fallax* was also cultivated by using [*methyl*-$^2H_3$]L-methionine containing medium [500 mL Middelbrook medium 7H9 supplemented with 0.468% (wt/vol) casitone, 0.032% (wt/vol) [*methyl*-$^2H_3$]L-methionine (99% $^2H_3$-enrichment) (Aldrich, Steinheim, Germany) and 1% (wt/vol) glucose].

*To whom correspondence should be addressed at Institut de Biologie Cellulaire et Génétique du CNRS, 118 Route de Narbonne 31032 Toulouse Cedex, France.

Abbreviations: ATCC, American Type Culture Collection; CAD, collision activated dissociation; CIP, collection of the Institut Pasteur; EI, electron impact ionization; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GC/MS/MS, gas chromatography/tandem mass spectrometry; MIKE(S), mass-analyzed ion kinetic energy (spectrum); MS/MS, tandem mass spectrometry; PFBE, pentafluorobenzyl ester.

Copyright © 1995 by AOCS Press

Isolation of nonhydroxylated fatty acid fractions. The cells were extracted by using a mixture of chloroform/methanol (2:1, vol/vol), and the dry extract was saponified in 1 mL of a 0.2 M solution of potassium hydroxide in a mixture ethanol/benzene/water (5:1:1, by vol) which was heated under reflux for 12 h at 85°C. Fatty acids were precipitated by a solution of sulfuric acid (0.2 M in water), extracted with diethyl ether and washed several times with water, then evaporated to dryness. Fatty acids were methylated with diazomethane in diethyl ether and chromatographed on an open, neutralized silicic acid column (CC7; Mallinckrodt Inc., St. Louis, MO) by using a gravity flow gradient of diethyl ether in hexane, adjusted by hand. The nonhydroxylated methylated fractions were pooled and analyzed by capillary GC.

Separation of saturated and unsaturated fatty acid methyl esters was achieved by silver nitrate (Sigma, St. Louis, MO) silicic chromatographic column as described by De Vries (23).

PFBE derivatization. Fatty acid methyl ester mixtures (0.5 mg) corresponding to each saturated and unsaturated fraction were saponified in 1 mL of 0.2 M KOH in water/methanol solution (1:1, vol/vol) at 80°C for 2 h. The PFBE derivatives of the mixture of liberated fatty acids were formed by treatment of 0.1–0.5 mg of each sample with pentafluorobenzyl bromide (2 μL) (Sigma) and diisopropyl methyl amine (2 μL) (Fluka, Buchs, Switzerland) in 60 μL of a mixture of dry methanol/acetonitrile (1:5, vol/vol). After 1 h at ambient temperature, the mixture was evaporated, dissolved in hexane (1 mL), and an aliquot analyzed by capillary GC.

GC experiments, GC/MS, and GC/MS/MS analysis. The gas chromatograph (Serie 30; Girdel, Dijon, France) was fitted with a solid injector (solvent split injection mode) and a fused silica capillary column (30 m × 0.32 mm i.d.) coated with nonpolar dimethyl silicon phase (OV1, 0.3-mm thickness; SGE, Ringwood, Victoria, Australia). The carrier gas was helium. The temperature program was 140–290°C at 2°C/min; the detector was a flame-ionization detector. The temperature of the injector was 240°C and, for the detector, 280°C.

GC/MS and GC/MS/MS analyses of PFBE were performed with a reverse geometry ZAB HS instrument (VG Analytical, Manchester, United Kingdom). It was fitted with a high-pressure ion source, and coupled with a capillary gas chromatograph (DANI 3800; Dani, Monza, Italy). The GC conditions were as described previously.

The electron capture ionization mode (identical to chemical ionization conditions) was obtained by using methane as moderator gas and a 50-eV electron beam. The pressure of methane was adjusted in order to have the maximum current of ion *m/z* 281 of electron capture ionization on 5 ng of pentafluorobenzyloleate (no pressure measurement in the ion source is available on the ZAB–HS instrument). The VG 2035 data system was used for acquisition and data processing.

For GC/MS/MS experiments, we have used the collision cell located between the magnet and the electrostatic analyzer. The pressure of helium was adjusted in order to reduce the main beam to approximately 50% of its original value. The MIKES were acquired in the continuum mode at a speed of 5000 $eV.s^{-1}$ from 7850 eV (0.98 E_0) to 400 eV (0.05 E_0) (E_0 is the value of the electric field transmitting the main beam). A 1.7-s cycle rate was obtained by using interscan delay of 0.2 s.

RESULTS AND DISCUSSION

Description of the method. Jensen *et al.* (12) have found that the decomposition of aliphatic ions containing a strongly localized electric charge (closed-shell ions) proceeds *via* a thermal-like mechanism with no participation of the charge (Scheme 1).

The mechanism involves a 1,4 loss of H_2 to give terminally unsaturated fatty acid carboxylates and 1-alkenes. In this process, double bonds do not migrate before fragmentation. Such a mechanism was found to operate in the CAD spectra of fatty acid carboxylates (10,12,14,16,17; for reviews see Refs. 24,25). Thus, the CAD spectra of the $(M - H)^-$ ion from saturated-chain fatty acids exhibit an envelope of regularly spaced signals at 14-mass-units intervals, owing to parallel cleavages of each C–C bond and simultaneous ejection of alkenes and hydrogen. When the aliphatic chain contains a double bond, allylic cleavages prevail, whereas cleavages of the double and vinylic bonds induce only weak signals. Thus, a "diagnostic" pattern of two intense signals separated by three weak ones indicates the presence of a double bond and allows its location by simply counting the number of signals between the main beam (the molecular ion mass) and the first weaker signal of the "dip," representing the three low-abundance ions. This gives the number of methylene groups between the methyl terminal and the double bond (10). A methylene branch is detected and located by the same phenomenon as double bonds, but there is a nearly complete absence of the middle single peak in a "dip" representing the three low-abundance ions (15,16). A methyl branch is detected and located by the absence of a single signal in the envelope (14).

Because the free fatty acids generally have less desirable chromatographic properties than their esters, we have gener-



SCHEME 1

$$e^- + R''\text{–}COO\text{–}CH_2\text{–}C_6F_5 \longrightarrow R''\text{–}COO^- + C_6F_5\text{–}CH_2^{\bullet}$$

SCHEME 2

ated gas-phase carboxylate anions from electron capture ionization of PFBE, according to Scheme 2 (19–21).

The chromatographic separation of such derivatives is possible for most positional isomers of monounsaturated, methyl branched, and cyclopropane-containing fatty acids, provided a good capillary column is used. The mass spectra are very simple, as carboxylate ions are the only ones produced. Carboxylate ions are formed in high yields by this ionization method (19–21). Because the collision activated processes of anions produce competing reactions of electron detachment (26,27) and ionic fragmentations (28), the intensity of the CAD spectra are generally weak. Nevertheless, the very high abundance of carboxylate anions obtained with electron capture ionization of PFBE allows their CAD/MIKE analysis to be performed by very fast kinetic-energy scans, in a reverse-geometry, double-focussing mass spectrometer.

A preliminary analysis of the fatty acids PFBE was carried out by conventional GC/MS. These experiments established the retention times of the various constituents and the masses of the corresponding carboxylate anions.

During the recording of GC/MS/MS analysis, just before the arrival of the chromatography peak, the magnetic field was adjusted to the value required for transmission of the carboxylate anion. Then, all spectra obtained by electric-field scanning were stored in the data system and transformed further into mass spectra. In the last part of the procedure, the whole acquired data were further converted into mass spectra by computer software. Thus, for each precursor ion of the same mass, we have obtained a reconstructed total (fragments plus parent) ion current chromatogram (GC/MS/MS chromatogram) and CAD spectra corresponding to the different chromatographic peaks of isomers. All of the GC/MS/MS chromatogram of each *m/z* value of precursor ion and CAD spectra obtained from these different precursor ion masses give the complete GC/MS/MS analysis.

Analysis of the fatty acids fractions of M. aurum *and* M. fallax. Fatty acid structures were easily determined with about 1.2 μg of fatty acid mixture. Saturated, unsaturated, odd, and even number fatty acids of *M. aurum* and *M. fallax* are described in Table 1. Three injections of each sample of PFBE fatty acid in GC/MS/MS were needed to make complete GC/MS/MS studies and to identify all the kinds of fatty acids.

The unsaturated fraction of *M. fallax* shows 12 major fatty acids, which represent about 98% of all the components of the fatty acid mixture (result estimated from gas chromatogram, not shown). Two percent of this mixture is composed of the 15 minor fatty acids reported in Table 1. We conclude that a mean of 1.6 nanograms (1200 ng × 0.02/15) of each PFBE fatty acid are needed for structural determination by GC/MS/MS.

Identification of three enzymatic C*-methylation processes in* M. fallax. Fatty acids of *M. fallax* were briefly reported by Levy-Frébault *et al.* (22). GC/MS/MS allows an exhaustive structural determination of these fatty acids.

Some years ago, it was shown that enzymatic C-alkylation process of isolated double bonds of unsaturated fatty acid is often observed in mycobacteria (29). Different reaction schemes were proposed in which different intermediate structures of fatty acids were described. These structures were very

TABLE 1
Saturated and Unsaturated Fatty Acids Identified from *Mycobacterium aurum* (American Type Culture Collection 10437) and *M. fallax* (Collection of the Institut Pasteur 8139)[a]

Fatty acid length	*M. aurum*		*M. fallax*	
	Saturated	Unsaturated	Saturated	Unsaturated
C_{14}	**n**, i	**Δ5**, ΔZ, Δ8, Δ10	**n**, i	ND
C_{15}	**n**, i, a, $\mathbf{R^6}$, R^2	ND	**n**, i, a, R^{10}, R^8	ND
C_{16}	**n**, i, R^7, R^2	Δ7, Δ9, **Δ10**	n	Δ7, **Δ9**, Δ10, Δ11, Δ13, Δ15
C_{17}	n, i, a, R^{10}, $\mathbf{R^8}$, R^2	ΔR^8, Δ9, Δ10, $\mathbf{\Delta 9 R^2}$	**n**, i, a, R^{12}, R^{10}, R^8, R^4	$\Delta 6R^{14}$, Δ8, $\Delta 8R^8$, **Δ9**, Δ13, ΔR^8, $C^{9,10}$
C_{18}	n, i, R^{10}, R^2, $R^{2,8}$	**Δ9**, $\Delta 9R^2$	**n**, R^{10}	**Δ9**, **Δ11**, Δ13
C_{19}	n, $\mathbf{R^{10}}$, R^2	$\mathbf{\Delta 9 R^2}$, ΔR^{10}	n, $\mathbf{R^{10}}$	$C^{11,12}$, Δ9, Δ10, ΔR^{10}, $\mathbf{\Delta 12 R^{11}}$
C_{20}	**n**, $R^{2,4}$	Δ9, **Δ11**, $\mathbf{\Delta 12 R^{2,4}}$	ND	Δ9, Δ10, Δ11, Δ12, Δ13
C_{22}	**n**, $R^{2,4}$	**Δ5**, Δ13	ND	ND
C_{24}	n	ND	ND	Δ15
C_{34}	ND	Δ4, 8, 12, 16	ND	ND
C_{36}	ND	Δ4, 8, 12, 16, 20	ND	ND

[a]Δ indicates the position of double bond; *i, iso* type; a, *anteiso* type; n, linear type; R^j, methyl branched on carbon j; ΔR^j, methylene branched on carbon number j; $R^{i,j}$, two methyl branched on carbon number i and j; $C^{i,j}$, cyclopropyl ring between carbon number *i* and j; Δ4, 8, 12, 16, tetraunsaturated on position 4, 8, 12, 16; Δ4, 8, 12, 16, 20, pentaunsaturated on position 4, 8, 12, 16, 20; ΔiR^j, unsaturated on position *i* and branched in position *j*; ND, nondetected. The major component in each series is bold, the second major component is underlined.

difficult to identify, and some of these fatty acids were never observed. We have focused our attention on the C_{18} and C_{19} saturated and unsaturated fatty acids from *M. fallax*, because there are implications for double-bond enzymatic methylation processes.

Figures 1–3 show the GC/MS/MS data of anion of *m/z* 297 (19:0) from the saturated fraction, another of *m/z* 295 (19:1) from the unsaturated fraction, and another of *m/z* 281 (18:1) from the unsaturated fraction. The top chromatogram in each figure represents the reconstructed total ion current profile from the fragments of each precursor ion. The subsequent spectra correspond to the CAD/MIKE spectra of the precursor ion at different GC retention times.

There are two principal components yielding anion of *m/z* 297 (Fig. 1A). The CAD spectra on the rising and falling sides of the main component 1B are all identical and show a dip of one signal at *m/z* 169, indicating a methyl branch on C_{10} carbon atom (Fig. 1B); it is assigned as 10-methyl-octadecanoic acid (tuberculostearic acid). Peak 1C of the GC/MS/MS chromatogram shows a small amount of a linear nonadecanoic acid (Fig. 1C).

The data for the anion *m/z* 295 (Fig. 2A) are more complex; there are approximately six fatty acids: Figure 2B (spectrum of peak *2B*) shows a dip of four weak signals between ions *m/z* 171 and 239, separated from the main beam by four intense signals. This was previously identified as a 11-methyl-12-octadecenoic acid (30). Figure 2C (spectrum of peak *2C*) is a 10-methyleneoctadecanoic acid (dip of three consecutive weak signals between ions *m/z* 141 and 197, extremely weak signal at *m/z* 155). Figure 2D, 2E, and 2F (spectra of peak *2C, 2D,* and *2E*) show, respectively, Δ9 (dip of three consecutive weak signals between ions *m/z* 127 and 183), Δ10 (dip of three consecutive weak signals between ions *m/z* 141 and 197), Δ11 (dip of three consecutive weak signals between ions *m/z* 155 and 211), nonadecenoic acid. Figure 2G (spectrum of peak *2G*) can be used to characterize clearly a 11,12-*C*-methylideneoctadecanoic acid (lactobacillic acid). Ions a, b, c, d, e, f, and g are diagnostic of a cyclopropyl ring, and ion c specifies its location at ω7 position (17).

The GC/MS/MS chromatogram of mass *m/z* 281 (18:1) (Fig. 3A) shows three GC peaks, which were identified as Δ9, Δ11-, and Δ13-octadecenoic acid (3B, 3C, 3D, spectrum of peaks *3B, 3C,* and *3D,* respectively).

These results identify three coexisting methylation processes in *M. fallax*. First, oleic acid is methylated into 10-methylene octadecanoic acid, followed by double-bond reduction to tuberculostearic acid. This mechanism was called the "CD_2 mechanism" some years ago, because two deuterium atoms were found on tuberculostearic acid when bacteria were cultivated on [*methyl*-$^2H_3$]L-methionine-containing medium (29,31–34, for a review see Ref. 35).

There are also two methylation processes of vaccenic acid: one gives lactobacillic acid (29,31–34) and the other produces 11-methyl-12-octadecenoic acid (37). Such methylation processes give two deuterium atoms in lactobacillic acid and three deuterium atoms in 11-methyl-12-octadecenoic acid, respectively, when bacteria are cultivated on [*methyl*-$^2H_3$]L-methionine-containing medium. This last process has been called the "CD_3 mechanism" (33–35,38).

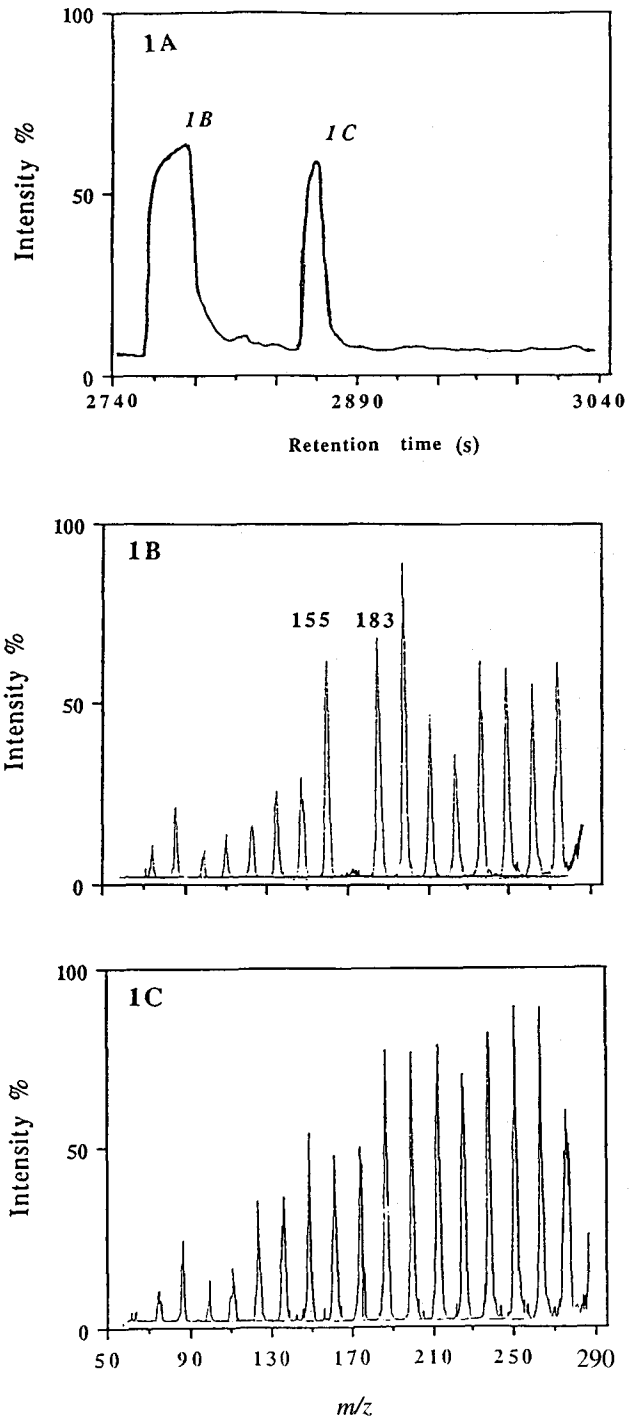


FIG. 1. Gas chromatography/tandem mass spectrometry analysis of saturated acids from *Mycobacterium fallax* (CIP 8139): nonadecanoic acid. A is the reconstructed chromatogram of anions of *m/z* 297. B and C are collision activated dissociation/mass-analyzed ion kinetic energy (CAD/MIKE) spectra of the two components labelled *1B* and *1C*, B, 10-methyloctadecanoic acid, C, *n*-nonadecanoic acid.

Table 2 gives the number of deuterium atoms found in branched fatty acids of *M. fallax* cultivated on [*methyl*-$^2H_3$]L-

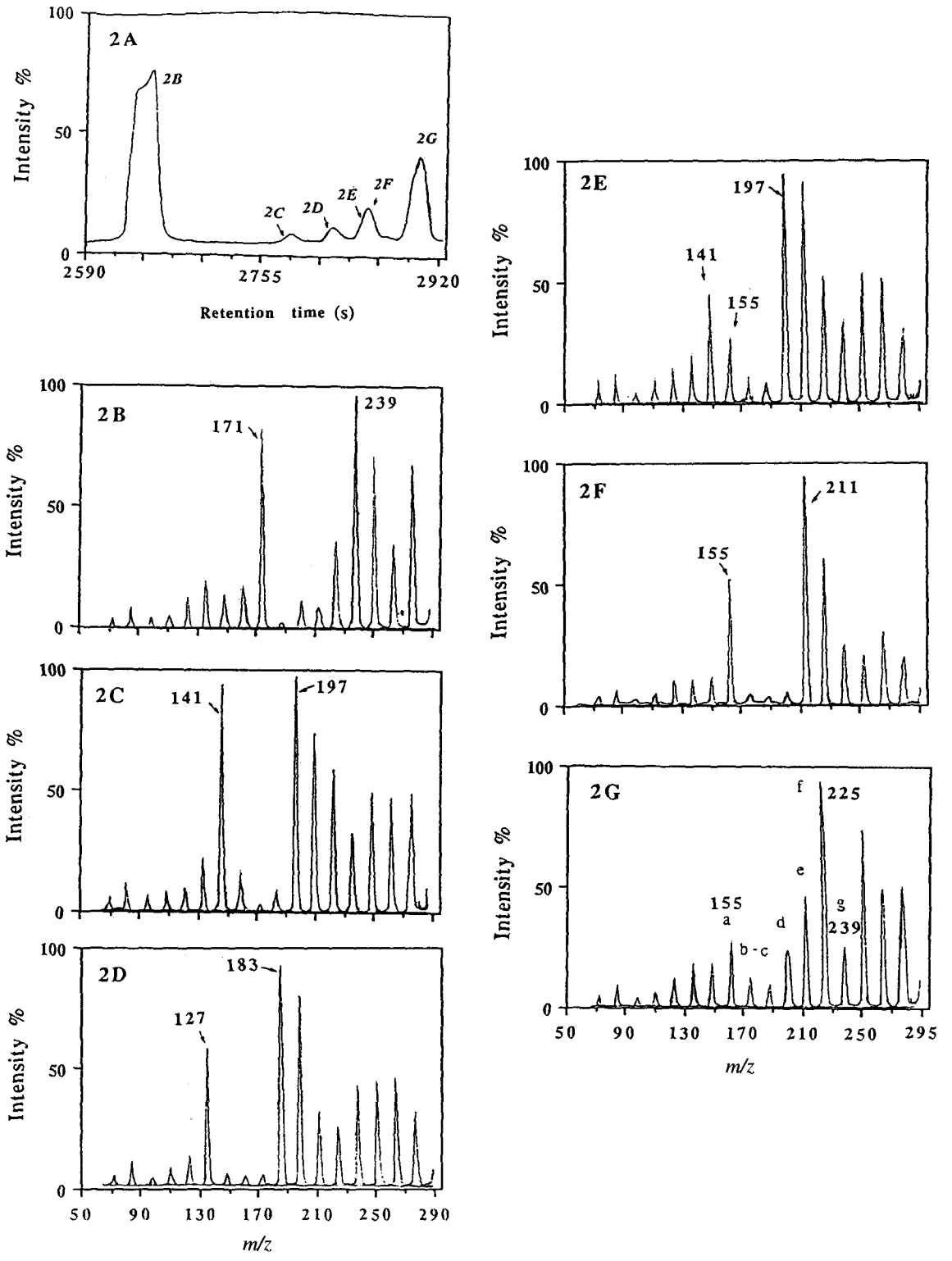


FIG. 2. Gas chromatography/tandem mass spectrometry (MS/MS) analysis of monounsaturated acid from *Mycobacterium fallax* (CIP 8139): nonadecenoic acid. A is the reconstructed chromatogram from MS/MS analysis of anion of *m/z* 295 ions. B–G: CAD/MIKE spectra of the components labelled *2B-2G*: 2B, 11-methyl-12-octadecenoic acid; 2C, 10-methyleneoctadecanoic acid; 2D, 9-nonadecenoic acid; 2E, 10-nonadecenoic acid, the abnormal high intensity of ion *m/z* 155 is due to the major ion of the following poorly separated fatty acid 2F; 2F, 11-nonadecenoic acid; and 2G, lactobacillic acid [ions noted a, b, c, d, e, f, and g are diagnostic ions of a cyclopropyl ring (Ref. 17)]. The mean quantity of each fatty acid is estimated to 1.6 ng. Abbreviations as in Figure 1.

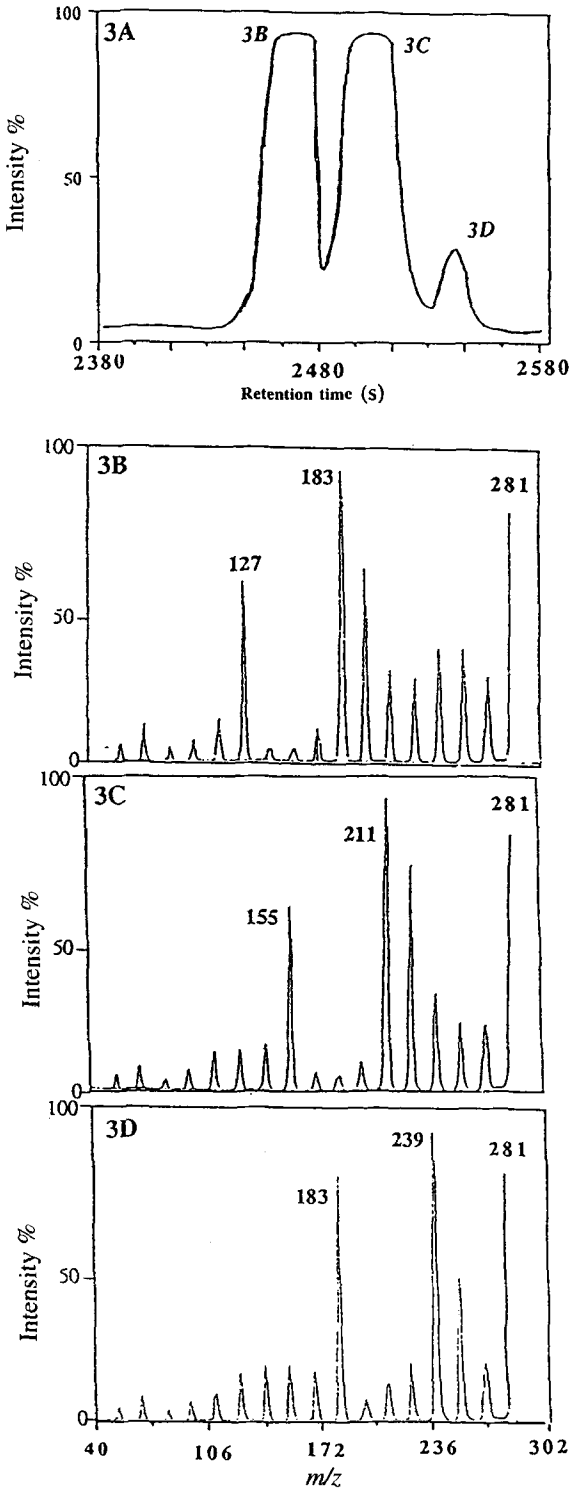


FIG. 3. Gas chromatography/MS/MS analysis of monounsaturated acids from *Mycobacterium fallax* (CIP 8139): octadecenoic acids. A is the reconstructed chromatogram of anions of *m/z* 281. 3B–3D, CAD/MIKE spectra of the three components, labelled *3B-3C*: 3B, 9-octadecenoic; 3C, 11-octadecenoic; and 3D, 13-octadecenoic. Abbreviations as in Figure 1.

methionine-containing medium, confirming the three methylation processes already described. The two deuterium atoms in 10-methyleneoctadecanoic acid and in tuberculostearic acid confirm the methylation process of oleic acid ("CD_2 mechanism"). Three deuterium atoms in 11-methyl-12-octadecenoic acid and two deuterium atoms in lactobacillic acid ("CD_3 mechanism") suggest two different and independent methylation processes for vaccenic acid. These are summarized in Scheme 3 (where ** indicates two deuterium atoms in fatty acid coming from Mycobacterium grown on [*methyl*-$^2H_3$]L-methionine-containing medium; and *** indicates three deuterium atoms in fatty acid coming from Mycobacterium grown on [*methyl*-$^2H_3$]L-methionine-containing medium).

TABLE 2
Number of Deuterium Atoms in Deuterated Fatty Acids from *Mycobacterium fallax* (Collection of the Institut Pasteur 8139) Grown on [*methyl*-$^2H_3$]L-Methionine-Containing Medium[a]

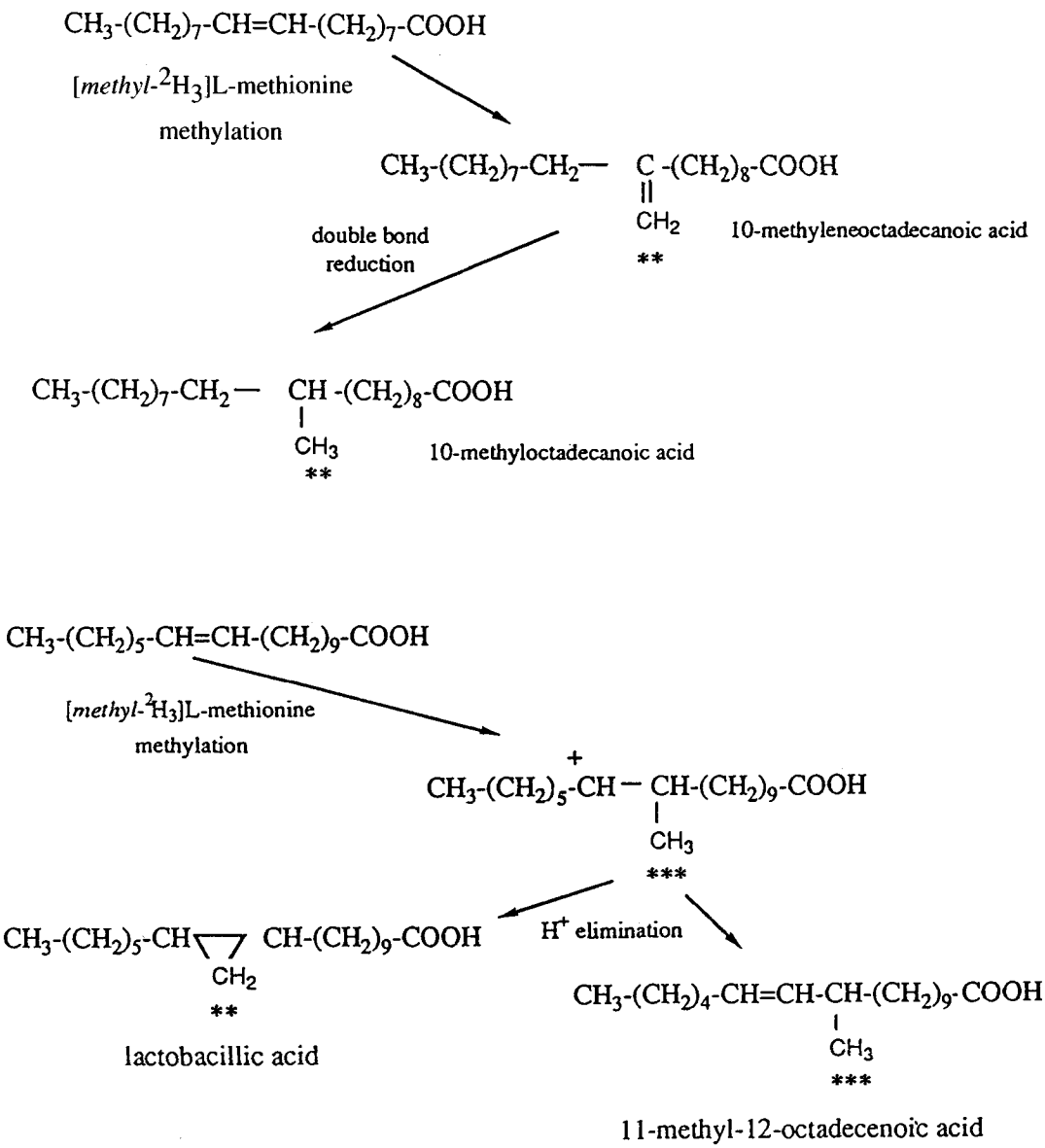
Fatty acid	Mass of labeled carboxylate ion (amu)	Mass of nonlabeled carboxylate ion (amu)	Number of D atoms in labeled fatty acid
11, 12-*C*-Methylidene octadecanoic	297	295	2
11-Methyl-12-octadecenoic	298	295	3
10-Methyl octadecanoic	299	297	2
10-Methylene octadecanoic	297	295	2

[a]These fatty acids are implicated in C methylation processes of oleic and vaccenic acids by methionine.

To my knowledge, this is the first report of the co-existence of these three C methylation processes on oleic and vaccenic acids in Gram-positive bacteria (38,39).

"Phleic acids" in M. aurum *fatty acids mixture. Mycobacterium aurum* fatty acids were described earlier (40,41), and contain many methyl branched fatty acids. We also identified two polyunsaturated fatty acids [phleic acids (42)]. CAD/MIKE spectra of the ion of *m/z* 525 (36:5) (Fig. 4, spectrum A) shows five dips of three weak signals between ions of *m/z* 58 to 111, 111 to 165, 165 to 219, 219 to 273, and 273 to 327, indicating a Δ4,8,12,16,20 36:5 fatty acid. The CAD/MIKE spectrum of the ion of *m/z* 471 (Fig. 4, spectrum B) indicates a Δ4,8,12,16 32:4 fatty acid. These polyunsaturated acids were previously described in *M. phlei* (43) and *M. smegmatis* (44).

Accurate analysis of fatty acid complex mixtures can be achieved by MS/MS of their carboxylate derivatives. The analysis can be obtained on very small quantities, down to nanogram levels. The instrumentation is relatively accessible because double-focussing sector reverse-geometry mass spectrometers are now commonplace. The experiments also can be done by linked scanning on a forward-geometry instrument, but only with difficulty on triple-quadruple tandem mass spectrometers (27). This technique is, therefore, a valuable addition to the tools of studying fatty acid biosynthetic pathways, the chemical taxonomy of bacterial strains, and all other fatty acid structural determination.



SCHEME 3

ACKNOWLEDGMENTS

I thank Conseil Régional Midi Pyrénées for financial support (BDI). I am also indebted to Professor G. Couderc for help in the preparation of this manuscript. Dr. J.C. Prome is thanked for the opportunity to carry out this work in his laboratory. I acknowledge the technical support given by Professor D. Promé, Dr. H. Aurelle, and A. Savagnac.

REFERENCES

1. Daffé, M., Lanéelle, M.A., Asselineau, C., Lévy-Frébault, V., and David H. (1983) *Ann. Microbial. (Inst. Pasteur) 134B,* 241–256.
2. Asselineau, J. (1982) *Ind. J. Chest Dis. 24,* 143–144.
3. McCloskey, J.A. (1970) in *Topics and Lipid Chemistry* (F.D. Gunstone, ed.) Vol. 1, pp. 369–388, Logos Press, London.
4. Minikin, D.E. (1978) *Chem. Phys. Lipids 21,* 313–347.
5. Anderson, B.A. (1978) *Prog. Chem. Fats Other Lipids 6,* 279–308.
6. Harvey, D.J. (1982) *Biomed. Mass Spectrom. 9,* 33–38.
7. Vetter, W., Meister, W., and Osterhelt, G. (1988) *Org. Mass Spectrom. 23,* 566–572.
8. Yu, Q.T., Zhang, J.Y., and Huang, Z.H. (1986) *Biomed. Environ. Mass Spectrom. 13,* 211–216.
9. Jensen, N.J., and Gross, M.L. (1987) *Mass Spectrom. Rev. 6,* 497–536.
10. Tomer, K.B., Crow, F.W., and Gross, M.L. (1983) *J. Am. Chem. Soc. 105,* 5487–5488.
11. Couderc, F., Berjeaud, J.M., and Promé, J.C. (1991) *Rapid Commun. Mass Spectrom. 5,* 92.
12. Jensen, N.J., Tomer, K.B., and Gross, M.L. (1985) *Anal. Chem. 57,* 2018–2021.
13. Aurelle, H., Treilhou, M., Promé, D., Savagnac, A., and Promé, J.C. (1987) *Rapid Commun. Mass Spectrom. 1,* 65–68.
14. Jensen, N.J., and Gross, M.L. (1986) *Lipids 21,* 362–365.
15. Promé, J.C., Aurelle, H., Couderc, F., Promé, D., Savagnac, A.,

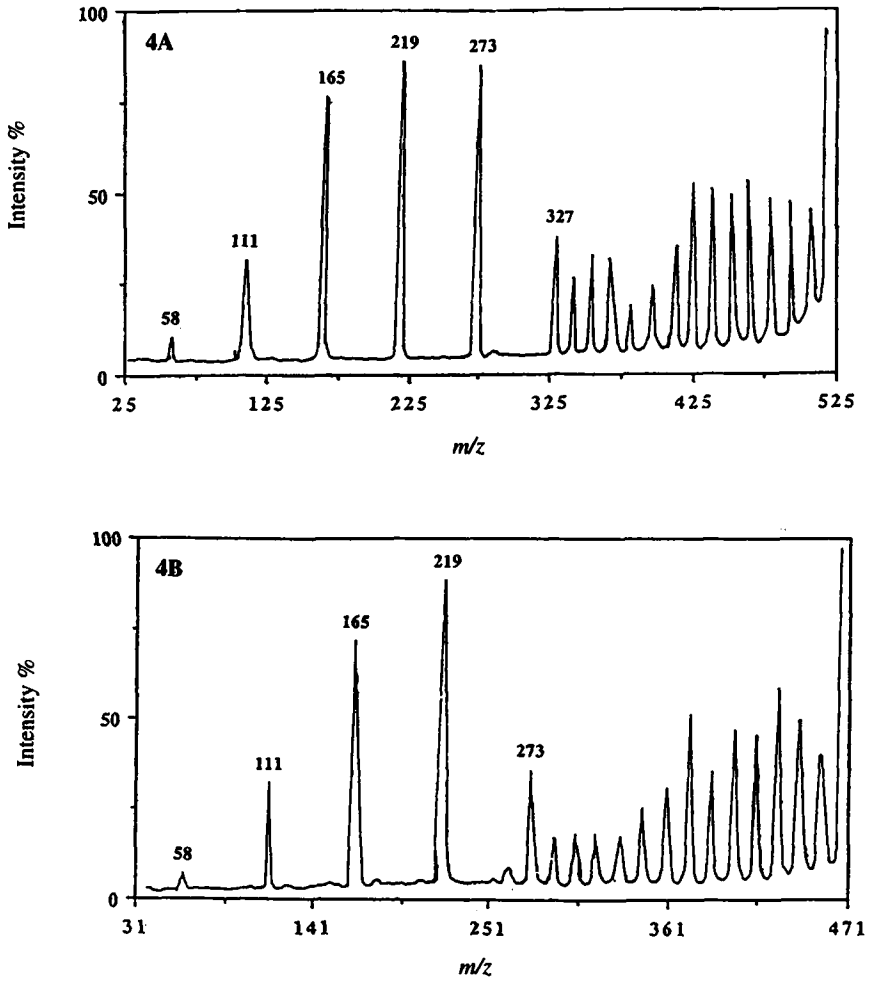


FIG. 4. CAD/MIKE spectra of the two polyunsaturated fatty acids from *Mycobacterium aurum* (American Type Culture Collection 10437). A, anion of *m/z* 525, 4,8,12,16-tetratriacontatetraenoic acid; B, anion of *m/z* 471, 4,8,12,16,20-hexatriacontapentaenoic acid. Abbreviations as in Figure 1.

and Treilhou, M. (1990) in *Analytical Microbiology Methods* (Fox, M., ed.) pp. 163–178, Plenum Press, New York.
16. Couderc, F., De Briel, D., Demont, N., Gilard, V., and Promé, J.C. (1991) *J. Gen. Microbiol. 137*, 1903–1909.
17. Tomer, K.B., Jensen, N.J., and Gross, M.L. (1986) *Anal. Chem. 58*, 2429–2433.
18. Le Quere, J.L., Sébédio, J.-L., Henry, R., Couderc, F., Demont, N., and Promé, J.C. (1991) *J. Chromatogr. 562*, 659–672.
19. Promé, J.C., Aurelle, H., Couderc, F., and Savagnac, A. (1987) *Rapid Commun. Mass Spectrom. 1*, 50–52.
20. Couderc, F., Aurelle, H., Promé, D., Savagnac, A., and Promé, J.C. (1989) *Biomed. Environ. Mass Spectrom.16*, 317–321.
21. Couderc, F. (1989) Applications Analytiques de Nouveaux Concepts en Spectrometry de Masse: Fragmentation d'Ions à Charge Piègée, FAB à Flot Continu, Ph.D. Thesis, Université Paul Sabatier, Toulouse, France, pp. 62–64.
22. Lévy-Frébault, V., Rafidinarivo, E., Promé, J.C., Grandry, J., Boisvert, H., and David, H. L. (1983) *Int. J. Syst. Bacteriol. 33*, 336–343.
23. De Vries, B. (1963) *J. Am. Oil Chem. Soc. 40*, 184–186.
24. Adams, J. (1990) *Mass Spectrom. Rev. 9*, 141–186.
25. Gross, M.L. (1992) *Int. J. Mass Spectrum. Ion Proc. 118/119*, 137–165.
26. Jensen, N.J., Tomer, K.B., and Gross, M.L. (1985) *J. Am. Chem. Soc. 107*, 1863–1868.
27. Tomer, K.B. (1989) *Org. Mass Spectrom. 24*, 969–972.
28. Graul, S.T., and Squires, R.R. (1988) *J. Am. Chem. Soc. 110*, 607–608.
29. Akamatsu, Y., and Law, J.H. (1970) *J. Biol. Chem. 245*, 701–708.
30. Couderc, F., Roche, P., Raynaud, C., Pougny, J.R., and Promé, J.C. (1989) *Adv. Mass Spectrom. 11B*, 1496–1497.
31. Jauréguiberry, G., Law, J.H., McCloskey, J.A., and Lederer, E. (1965) *Biochemistry 4*, 347–353.
32. Lanéelle, G., and Asselineau, J. (1990) in *Biochimie Bactérienne* (Hermann, F., ed.) pp. 87–88, Editeur des sciences et des arts, Paris.
33. Jauréguiberry, G., Lenfant, M., Das, B.C., and Lederer, E. (1966) *Tetrahedron, Suppl. 8*, 27–32.
34. Lenfant, M., Audier, H., and Lederer, E. (1966) *Bull. Soc. Chim. Fr. 9*, 2775–2777.
35. Lederer, E. (1969) *Quart. Rev. 27*, 453–481.

36. Liu, T.Y., and Hofmann, K. (1962) *Biochemistry 1*, 189–191.
37. Spencer, G.F., Payne-Wahl, K., and Plattner, R.D. (1979) *Lipids 14*, 72–74.
38. Lederer, E., and Azerad, R. (1973) *Biochimie des lipides* (McGraw-Hill, J.F., ed.) pp. 3–58, Edisciences, Paris.
39. Lechevalier, M.P. (1977) *CRC Microbiol. 11*, 109–210.
40. Rafidinarivo, E., Savagnac, A., Lacave, C., and Promé, J.C. (1982) *Biochim. Biophys. Acta 711*, 266–271.
41. Valero-Guillen, P.L., Pacheco, F., and Martin-Luengo, F. (1985) *J. Appl. Bacteriol. 59*, 113–126.
42. Aurelle, H., Couderc, F., Gavard, P., Promé, D., Treilhou, M., and Promé, J.C. (1989) *Adv. Mass Spectrom. 11B*, 1342–1343.
43. Asselineau, C., Montrozier, H., and Promé, J.C. (1969) *Eur. J. Biochem. 10*, 580–584.
44. Laslo, A., and Eidus, L. (1978) *Int. J. System. Bacteriol. 28*, 602–604.

[Received October 28, 1993, revision received April 28, 1995; Revision accepted May 18, 1995]

Peroxisomes in Mice Fed a Diet Supplemented with Low Doses of Fish Oil

C. Van den Branden[a,*], D. De Craemer[a], M. Pauwels[a] and J. Vamecq[b]

[a]Menselijke Anatomie & Embryologie, Vrije Universiteit Brussel, 1090 Brussels, Belgium and [b]North France Center for the Study of Childhood Epilepsy, 59037 Lille Cedex, France

ABSTRACT: The influence of low dietary doses (0.1 and 0.8% w/w) of a commercial fish oil preparation on peroxisomes in normal mice was studied and compared to the known strong inductive effects of high (10%) fish oil diets. Low fish oil doses were chosen to supply the mice with a concentration of docosahexaenoic acid, which was beneficial to patients with a peroxisomal disease. Peroxisomes were evaluated by cytochemical, morphometric, and enzymological techniques. The 0.1% fish oil diet had no effect on peroxisomes in liver, heart, and kidney even after prolonged treatment. The 0.8% diet did not change the peroxisomal number nor the catalase (EC 1.11.1.6) activity in the liver. Hepatic peroxisomal β-oxidation, however, was increased by 50% after 14 d. This was accompanied by reduced peroxisomal size. The 0.8% diet also caused a small increase (+25%) in myocardial catalase activity. No effect was observed in kidneys. Our results indicate that in mice a low (<0.8%) dietary fish oil dose has no or only a slight effect on hepatic peroxisomal β-oxidation. This may be of particular interest to patients with a peroxisomal fatty acid β-oxidation defect and who display a severe deficiency of docosahexaenoic acid—diets supplemented with low fish oil doses will improve the docosahexaenoic acid level without adding a strong load to the disturbed fatty acid metabolism.

Lipids 30, 701–705 (1995).

Peroxisomes are cell organelles with multiple functions (1). Their importance was revealed when Goldfischer *et al.* (2) reported their absence in the liver and the kidneys of patients with Zellweger's syndrome. A number of other diseases also could be related to the absence of peroxisomes or to a deficiency of one or more peroxisomal enzymes (3). Today there is no established therapy for these diseases. Therapeutic approaches range from dietary treatment to organ transplantation (4).

Recently it was found that patients with several peroxisomal disorders show depletion of docosahexaenoic acid (DHA) in brain, retina, liver, kidney, and erythrocytes (5,6). DHA ethyl ester has been added to the diet of two children diagnosed with neonatal adrenoleukodystrophy (NALD). In both patients, biochemical changes were observed (normalization of erythrocyte n-3 polyunsaturated fatty acids and increase in the 18:0 molecular species of plasmalogens); in one patient, a one-year administration resulted in significantly decreased 24:1n-9/22:0 and 26:1n-9/22:0 ratios and clear neurologic and electrophysiologic improvement (7,8). For this reason, Martinez *et al.* (8) suggested a dietary treatment with DHA ethyl ester to all patients with lowered blood levels of DHA. This suggestion was supported by Roels *et al.* (9).

In a study on the influence of a commercial fish oil preparation containing 20% DHA and 30% EPA (eicosapentaenoic acid) on peroxisomal enzymes and morphology, we concluded that a 10% (w/w) fish oil diet had a strong stimulatory effect on peroxisomal metabolism in mouse liver and myocardium (10,11). The final concentration of DHA present in this diet was 2% and corresponds to a daily intake of 3.5 g/kg body weight of the mice. This is high when compared to the daily oral dose of 250 mg DHA ethyl ester administered to the children with NALD (8). Two low doses of Bęromegan (a gift from FHER, Boehringer Ingelheim, Germany) were chosen in order to supply the mice with DHA concentrations comparable to those clinically tested. The aim of this study was to investigate the possible effects of these low doses of Beromegan on peroxisomes in liver, heart, and kidneys of healthy mice and to compare them to the findings previously obtained after treatment with a 10% fish oil diet (10,11).

MATERIALS AND METHODS

Adult male NMRI mice (35–40 g) were randomly assigned into groups of at least five animals. They were fed a standard laboratory meal (A04; UAR, Epinay, France) supplemented with 0.1% Beromegan for 3, 21, and 100 d or with 0.8% Beromegan for 3 and 14 d. Beromegan is a fish (salmon) oil extract mainly containing polyunsaturated fatty acids. The composition of the standard laboratory meal and of Beromegan is given in Table 1. The dietary doses were chosen to approximate the daily oral dose of 250 mg DHA ethyl ester administered in a clinical setting to the two children with NALD (8). We considered the body surface area as reference point to convert doses from human to mice (12), and calculated that a dose of 250 mg/d given to a child weighing a maximum 20 kg corresponds to a daily intake of 104 mg/kg body

*To whom correspondence should be addressed at Vrije Universiteit Brussel, Menselijke Anatomie & Embryologie, Laarbeeklaan 103, B-1090 Brussels, Belgium.

Abbreviations: DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); NALD, neonatal adrenoleukodystrophy.

Copyright © 1995 by AOCS Press

TABLE 1
Composition of Diet

Standard laboratory meal[a]		Beromegan®[b]	
Compound	% of Total	Fatty acid	% of Total
Moisture	12	14:0	6
Proteins	17	16:0	13
Sugar	58.7	16:1	8
Cellulose	4.3	16:2	1
Minerals	5	17:0	1
Lipids:	3 (=100%)	18:0	3
16:0	10.1	18:1	10
16:1	trace	18:2	1.5
18:0	2.0	18:4	1
18:1	31.1	20:0	1
20:2	56.4	20:4	0.5
18:3	trace	20:5	30
		22:4	1
		22:5	2
		22:6	20

[a]With details of fatty acids present. Composition from UAR (Epinay, France).
[b]Besides fatty acids, Beromegan® also contains ± 0.025% α-tocopherol and 1% cholesterol. Fatty acid composition from FHER (Boehringer Ingelheim, Germany).

weight in the mouse. The intake of DHA by the mice on a diet with 0.1 or 0.8% Beromegan was 35 mg/kg/d and 280 mg/kg/d, respectively, being ± one-third and three-fold, respectively, of the clinical dose. In comparison, the 10% Beromegan diet used in previous studies (10,11) corresponds to a daily uptake of 3500 mg DHA/kg body weight of the mice. The time control groups received the A04 meal only. All animals had free access to food and water.

Peroxisomal fatty acyl–CoA oxidase (EC 1.3.99.3) activity in liver (first- and rate-limiting step of the peroxisomal β-oxidation) was studied using the method of Vamecq (13), the cyanide-insensitive fatty acyl–CoA oxidation in liver (third step of the peroxisomal β-oxidation) was measured as described by Lazarow and de Duve (14), and catalase (EC 1.11.1.6) activity was assayed in homogenates of liver, heart, and kidney according to Baudhuin (15) and Van den Branden *et al.* (16).

Histological analysis (after diaminobenzidine staining for catalase) and morphometry of hepatic peroxisomes were performed as previously described (17). All results are given as the mean ± SEM. For statistical analysis, the Mann–Whitney test was used (18). Significance was obtained when $P < 0.05$.

RESULTS

The fish oil diet was well accepted by the mice. At the end of the experiment, body weight of Beromegan-treated animals was similar to the weight of control animals. No hepatomegaly was observed. Liver, heart, and kidney weights expressed as percentages of body weight were not changed. The time control groups showed no differences in peroxisomal β-oxidation capacity, catalase activity, or morphometric parameters and, therefore, were pooled into one control group.

The biochemical data, as well as enzymological data obtained in a previous experiment using a 10% fish oil diet are included in Table 2 to facilitate observation of dose- and time-related changes. Even a prolonged treatment of 100 d with 0.1% fish oil failed to induce peroxisomal β-oxidation. In animals fed a diet supplemented with 0.8% Beromegan for 14 d, peroxisomal β-oxidation was significantly increased (± ×1.5). Previous experiments revealed a four-to-fivefold increase of peroxisomal β-oxidation capacity in mice fed a diet supplemented with a high dose (10%) of Beromegan after only three days of treatment (Table 2) (10,11).

Hepatic catalase activity was not changed in mice fed the diets supplemented with 0.1 or 0.8% fish oil. Even after 100 d of 0.1% Beromegan administration, catalase activity was similar to values in control animals (Table 2). Renal catalase activity was also unchanged by the fish oil diets (results not shown). Myocardial catalase activity was significantly increased only in mice fed the 0.8% Beromegan diet—when compared to controls (1.26 ± 0.07 U_B/g), catalase activity was 1.60 ± 0.06 U_B/g and 1.55 ± 0.12 U_B/g after 3 and 14 d of treatment, respectively ($P < 0.05$).

Light microscopic study of diaminobenzidine-stained liver, heart, and kidney sections did not reveal differences in number, size, and staining intensity of the peroxisomes between control and Beromegan-fed animals. Apparently, the increase in myocardial catalase activity in animals fed the

TABLE 2
Effects of Different Dietary Doses of Beromegan[a] on the Activity of Catalase and the First and Third Step of Peroxisomal β-Oxidation in the Liver of Mice

		0.1% Beromegan			0.8% Beromegan		10% Beromegan		
Enzyme	Control	3 d	21 d	100 d	3 d	14 d	3 d	14 d	21 d
Catalase[b]	88 ± 4	87 ± 2	85 ± 2	80 ± 3	83 ± 2	86 ± 5	136 ± 5[c]	157 ± 5[c]	161 ± 6[c]
Lauroyl–CoA oxidase[d]	7.6 ± 0.3	7.1 ± 0.7	7.1 ± 0.9	7.7 ± 0.9	ND	12.7 ± 0.6[c]	40.8 ± 1.4[c]	56.6 ± 1.6[c]	57.9 ± 1.8[c]
Palmitoyl–CoA oxidase[d]	5.2 ± 0.3	4.3 ± 0.4	4.2 ± 0.8	5.0 ± 0.5	ND	7.3 ± 0.4[c]	21.9 ± 1.0[c]	33.2 ± 1.2[c]	34.0 ± 0.6[c]
Cyanide-insensitive lauroyl–CoA oxidation[e]	6.6 ± 0.4	6.1 ± 0.6	6.2 ± 0.8	6.6 ± 0.8	ND	11.2 ± 0.4[c]	37.2 ± 1.1[c]	52.5 ± 0.9[c]	54.3 ± 1.2[c]
Cyanide-insensitive palmitoyl-CoA oxidation[e]	4.4 ± 0.3	3.5 ± 0.8	3.3 ± 0.8	4.1 ± 0.4	ND	6.0 ± 0.4[c]	19.9 ± 1.2[c]	31.0 ± 1.3[c]	31.8 ± 0.4[c]

[a]Company source as in Table 1. ND, not done.
[b]UB/g.
[c]Significantly different from control value (P at least <0.05).
[d]nmol H_2O_2 per minute and mg protein.
[e]nmol NAD_+ reduced per minute per mg protein.

TABLE 3
Morphometry of Hepatocellular Peroxisomes in Mice Fed Different Doses of Beromegan[a]

	Control	0.1% Beromegan			0.8% Beromegan
		3 d	21 d	100 d	14 d
D-circle (μm)	0.382 ± 0.006	0.398 ± 0.005	0.376 ± 0.017	0.376 ± 0.008	0.354 ± 0.011[b]
Volume density (%)	1.641 ± 0.146	1.551 ± 0.217	1.561 ± 0.149	1.833 ± 0.071	1.613 ± 0.139
Numerical density (μm^{-3})	0.433 ± 0.037	0.354 ± 0.046	0.494 ± 0.083	0.524 ± 0.053	0.553 ± 0.046
Surface density (μm^{-1})	0.240 ± 0.020	0.222 ± 0.031	0.249 ± 0.022	0.278 ± 0.015	0.262 ± 0.017

[a]Company source as in Table 1.
[b]Significantly different from control value ($P < 0.05$).

0.8% Beromegan diet was too small to be noticed by visual evaluation of catalase stained myocardial sections.

Ultrastructural investigation of the liver sections of fish oil-fed mice failed to reveal differences in electron density of the peroxisomal matrix (catalase reaction product) in size or in number of peroxisomes when compared to control liver sections. Peroxisomal shape strongly varied in all control and treated mouse livers. Morphometric analysis (Table 3) demonstrated a decreased peroxisomal size in 0.8% fish oil-fed mice after 14 d. This was not observed in mice fed the 0.1% Beromegan diet, not even in the animals fed this diet for 100 d. No differences in volume density, numerical density, or surface density were found with either diet.

Myocardial peroxisomes contained a reticular matrix in both control and in fish oil-fed mice. The electron density of the peroxisomal matrix showed no differences between the experimental groups and the control animals. The organelles were strongly dispersed and sparse. By visual evaluation, size, shape, and number were unchanged.

DISCUSSION

We recently showed that a high fish oil diet containing 10% Beromegan induces a strong (up to eightfold) increase in peroxisomal β-oxidation capacity and a twofold increase in catalase activity and peroxisomal number in the liver (Table 2) (10). In the present study, we found a moderate (up to 1.7-fold) increase in peroxisomal β-oxidation capacity and a reduction of the peroxisomal size in the liver of mice fed a 0.8% fish oil diet, while a lower dose (0.1%) of fish oil did not influence the investigated peroxisomal parameters. These findings indicate that more and stronger peroxisomal changes are observed with increasing dietary fish oil concentrations. The duration of dietary treatment is also an important factor—the increase in peroxisomal β-oxidation capacity provoked by the 10% fish oil diet was already evident after three days (10,11), the 0.8% fish oil diet induced only a moderate increase after 14 d, and the 0.1% fish oil diet failed to affect peroxisomal β-oxidation after up to 100 d of treatment.

To our knowledge, papers reporting on peroxisomal characteristics in animals fed a diet supplemented with concentrations of fish oil as low as in our present experiments are not available. A significant increase in the activities of catalase and peroxisomal fatty acyl–CoA oxidase was found in the liver of rats fed diets with different n-3/n-6 fatty acid ratios (ranging between 0.12 and 10) (19). However, the total amount of fatty acids in each diet was 10% (w/w) (19,20), and this is considerably higher than in the present experiments. In our experiments, the animals fed the 0.8% fish oil diet had a daily intake of 280 mg DHA/kg body weight and 420 mg EPA/kg body weight; the daily ingestion of all polyunsaturated fatty acids amounted to ±800 mg/kg body weight. Demoz *et al.* (21) demonstrated an increase of hepatic peroxisomal acyl–CoA oxidase and catalase activities of 50 and 30%, respectively, after daily administration of 1000 mg ethyl–EPA/kg body weight to mice for 10 d. In rats, the same daily intake of ethyl–EPA for three months had a similar effect on both peroxisomal enzymes (22). Ethyl-DHA, administered in a daily dose of 1000 mg/kg body weight for three months to rats, induced the fatty acyl–CoA oxidase and catalase activities in the liver (+64 and +18%, respectively) (22). In addition, peroxisomal β-oxidation and fatty acyl–CoA oxidase activity were significantly increased in the peroxisome-enriched fraction of the liver of rats on a daily intake of 1000 mg ethyl–EPA or ethyl–DHA/kg body weight for 10 d (23). In our experiments, the lower dose of n-3 polyunsaturated fatty acids in the 0.8% fish oil diet, as well as the use of mice instead of rats as experimental animals, may account for the slightly weaker induction of the peroxisomal β-oxidation capacity and the normal catalase activity (Table 2) when compared to the findings of Demoz *et al.* (21,22) and Willumsen *et al.* (23). In this respect, we assume that the increased production of H_2O_2 by peroxisomal fatty acyl–CoA breakdown in response to the 0.8% Beromegan diet is not sufficiently high to require an increased activity of catalase in order to avoid cell damage.

It is often noticed that a strong induction of the peroxisomal β-oxidation capacity is not accompanied by as strong an increase in catalase activity. In fact, it represents a constant finding in male rodents after treatment with one of the so-called peroxisome proliferators (24). From our present and previous experiments, we calculated the ratio of induction of peroxisomal β-oxidation to catalase (Table 4). The imbalance between the induction of a peroxisomal H_2O_2-generating metabolic pathway (fatty acid β-oxidation) and catalase, the most important H_2O_2-metabolizing peroxisomal enzyme, created by a dietary treatment of 10% fish oil, mimics the effect of a peroxisome proliferator. The resulting increase in peroxisomal H_2O_2-pool may be detrimental to cellular function and DNA, and is believed to be the underlying mechanism of the

TABLE 4
Changes in Peroxisomal β-Oxidation Capacity and Catalase Activity in the Liver of Mice on Diets Supplemented with Different Doses of Fish Oil

	% of the control		
Enzyme activities	0.1% Fish oil for 100 d	0.8% Fish oil for 14 d	10% Fish oil for 14 d
Cyanide-insensitive β-oxidation	94%	154%[a]	740%[a]
Catalase	91%	98%	178%[a]
β-Oxidation/catalase	1.0	1.6	4.2

[a]Statistically significant.

induction of hepatic neoplasia in rodents treated with peroxisome proliferators (25,26). However, the effective concentration of any peroxisome proliferator to obtain this metabolic imbalance is far below the fish oil dose needed. In addition, peroxisome proliferators also induce specific changes in peroxisomal morphology in the liver of susceptible animals (male rodents)—an increase in size and number, sometimes resulting in a 20-fold increase of total peroxisomal volume (27). In contrast, the 10% Beromegan diet induced a doubling of the peroxisomal volume density and did not change the mean size of the organelles (10). These differences in effective dose and peroxisomal effects may be a result of different influences on other enzyme activities. A stimulation of peroxisomal metabolism in the liver in conditions other than a treatment with peroxisome proliferators is often accompanied by an increase in number and a reduction in size of the peroxisomes (10). In the liver of mice fed the 0.8% Beromegan diet, a significant reduction in size was found when the numerical density was [not significantly ($P = 0.16$)] increased with 27%. In addition, the lowest tested dose did not provoke peroxisomal fatty acid oxidation alterations or changes in peroxisomal size and number.

No changes in catalase activity or in intensity of the catalase staining of the peroxisomes were observed in the kidney with either diet. This is in line with the findings previously made in mice fed a 10% fish oil diet (10,11).

Catalase activity was not changed in mice myocardium on the 0.1% fish oil diet; it was slightly but significantly increased in mice fed the 0.8% fish oil diet, and this after three days. Catalase activity was more pronounced in mice fed a 10% fish oil diet (10). This indicates that myocardial response is dose-dependent. Increased catalase activity may point to an increased production of H_2O_2. Diets supplemented with partially hydrogenated marine oils or with soybean oils provoked an increase in catalase activity, together with an increase in the H_2O_2-generating peroxisomal fatty acid β-oxidation (28). Myocardial and hepatic response to Beromegan are different with respect to catalase induction. The effect of drugs and dietary fatty acids on peroxisomal metabolism in myocardium can be very different from what is found in liver (29,30).

Our results indicate that a dietary supplementation of low doses of DHA and EPA has little effect on the hepatic peroxisomal compartment in healthy mice. Only the 0.8% Beromegan diet induces an adaptation of the peroxisomal morphology, permitting a more efficient metabolism, as is reflected in a significant increase in β-oxidation activity. A 50% increase of peroxisomal β-oxidation can be considered as only a minor change when compared to the effects of the high-fat diets (10) or peroxisome proliferators (24). Though we cannot extrapolate mouse results to peroxisomal patients, we would like to make the following reflection—patients with Zellweger's syndrome, pseudo-Zellweger's syndrome, and typical or atypical NALD suffer from a defective peroxisomal fatty acid β-oxidation (4) and reveal a severe deficiency of DHA (6,8). The administration of low doses of fish oil [comparable with the 0.1% (w/w) dose we tested] most probably will not cause an additional load to the already disturbed hepatic peroxisomal fatty acid β-oxidation metabolism in these patients; on the other hand, recent evidence (8) has shown that peroxisomal patients will benefit from the supply of DHA to correct for the DHA deficiency in brain, retina, and in several other organs. Efforts to induce peroxisomes in patients with Zellweger's syndrome were not successful (31). DHA treatment is especially recommended for patients with a peroxisomal biogenesis defect (8,9). The fact that the DHA doses which give clinical improvement are low enough not to be a burden for β-oxidation can be considered an advantage.

ACKNOWLEDGMENTS

This research was supported by grant 2923220790 from the Belgian National Fonds voor Wetenschappelijk Onderzoek and grant 194.332.1500 from the Onderzoeksraad-Vrije Universiteit Brussel. JV is Chargé de Recherche of the French INSERM (Institut National de la Santé et de la Recherche Médicale).

REFERENCES

1. van den Bosch, H., Schutgens, R.B.H., Wanders, R.J.A., and Tager, J.M. (1992) Biochemistry of Peroxisomes, *Annu. Rev. Biochem. 61*, 157–197.
2. Goldfischer, S., Moore, C.L., Johnson, A.B., Spiro, A.J., Valsamis, M.P., Wisniewski, H.M., Ritch, R.H., Norton, W.T., Rapin, I., and Gartner, L.M. (1973) Peroxisomal and Mitochondrial Defects in the Cerebro-Hepatorenal Syndrome, *Science 182*, 62–64.
3. Wanders, R.J.A., Schutgens, R.B.H., Barth, P.G., Tager, J.M., and van den Bosch, H. (1993) Postnatal Diagnosis of Peroxisomal Disorders: A Biochemical Approach, *Biochimie (Paris) 75*, 269–279.
4. Brown, F.R., Voigt, R., Singh, A.K., and Singh, I. (1993) Peroxisomal Disorders. Neurodevelopmental and Biochemical Aspects, *Am. J. Dis. Child. 147*, 617–626.
5. Martinez, M. (1989) Polyunsaturated Fatty Acid Changes Suggesting a New Enzymatic Defect in Zellweger Syndrome, *Lipids 24*, 261–265.

6. Martinez, M. (1992) Abnormal Profiles of Polyunsaturated Fatty Acids in the Brain, Liver, Kidney and Retina of Patients with Peroxisomal Disorders, *Brain Res. 583*, 171–182.
7. Martinez, M. (1992) Treatment with Docosahexaenoic Acid Favorably Modifies the Fatty Acid Composition of Erythrocytes in Peroxisomal Patients. *Prog. Clin. Biol. Res. 375*, 389–397.
8. Martinez, M., Pineda, M., Vidal, R., Conill, J., and Martin, B. (1993) Docosahexaenoic Acid—A New Therapeutic Approach to Peroxisomal-Disorder Patients: Experience with Two Cases, *Neurology 43*, 1389–1397.
9. Roels, F., Fischer, S., and Kissling, W. (1993) Polyunsaturated Fatty Acids in Peroxisomal Disorders: A Hypothesis and a Proposal for Treatment, *J. Neurol. Neurosurg. Psychiat. 56*, 937.
10. De Craemer, D., Vamecq, J., Roels, F., Vallée, L., Pauwels, M., and Van den Branden, C. (1994) Peroxisomes in Liver, Heart and Kidney of Mice Fed a Commercial Fish Oil Preparation: Original Data and Review on Peroxisomal Changes Induced by High-Fat Diets, *J. Lipid Res. 35*, 1241–1250.
11. De Craemer, D., Roels, F., and Van den Branden, C. (1993) Rapid Effects of Dietary Fish Oil on Peroxisomes in Mouse Liver, *Eur. J. Morphol. 30*, 331–335.
12. Devita, Jr., V.T. (1989) Principles of Chemotherapy in *Cancer, Principles and Practice of Oncology* (Devita, V.T., Hellman, S., and Rosenberg, S.A., eds.), pp. 276–300, J.B. Lippincott, Philadelphia.
13. Vamecq, J. (1990) Fluorometric Assay of Peroxisomal Oxidases, *Anal. Biochem. 186*, 340–349.
14. Lazarow, P.B., and de Duve, C. (1976) A Fatty Acyl–CoA Oxidizing System in Rat Liver Peroxisomes: Enhancement by Clofibrate, a Hypolipidemic Drug, *Proc. Natl. Acad. Sci. USA 73*, 2043–2046.
15. Baudhuin, P (1974) Isolation of Rat Liver Peroxisomes, *Methods Enzymol. 31*, 358–368.
16. Van den Branden, C., Kerckaert, I., and Roels, F. (1984) Peroxisomal ß-Oxidation from Endogenous Substrates. Demonstration Through H_2O_2 Production in the Unanesthetized Mouse, *Biochem. J. 218*, 697–702.
17. De Craemer, D., Kerckaert, I., and Roels, F. (1991) Hepatocellular Peroxisomes in Human Alcoholic and Drug-Induced Hepatitis: A Quantitative Study, *Hepatology 14*, 811–817.
18. Snedecor, G.W., and Cochran, W.G. (1980) *Statistical Methods*, 7th edn., pp. 135–148, Iowa State University Press, Ames.
19. Vamecq, J., Vallée, L., Lechêne de la Porte, P., Fontaine, M., De Craemer, D., Van den Branden, C., Lafont, H., Grataroli, R., and Nalbone, G. (1993) Effect of Various n-3/n-6 Fatty Acid Ratio Contents of High Fat Diets on Rat Liver and Heart Peroxisomal and Mitochondrial ß-Oxidation, *Biochim. Biophys. Acta 1170*, 151–157.
20. Grataroli, R., Vamecq, J., Poupaert, J.H., Léonardi, J., Termine, E., Lafont, H., and Nalbone, G. (1992) Effects of Dietary n-6/n-3 Ratios on Lipid and Prostaglandin E2 Metabolism in Rat Gastric Mucosa, *J. Lipid Mediators 5*, 227–236.
21. Demoz, A., Willumsen, N., and Berge, R.K. (1992) Eicosapentaenoic Acid at Hypotriglyceridemic Dose Enhances the Hepatic Antioxidant Defense in Mice, *Lipids 27*, 968–971.
22. Demoz, A., Asiedu, D.K., Lie, Ø., and Berge, R.K. (1994) Modulation of Plasma and Hepatic Oxidative Status and Changes in Plasma Lipid Profile by n-3 (EPA and DHA), n-6 (corn oil) and a 3-Thia Fatty Acid in Rats, *Biochim. Biophys. Acta 1199*, 238–244.
23. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M., and Berge, R.K. (1993) Docosahexaenoic Acid Shows no Triglyceride Lowering Effects But Increases the Peroxisomal Fatty Acid Oxidation in Liver of Rats, *J. Lipid Res. 34*, 13–22.
24. Bentley, P., Calder, I., Elcombe, C., Grasso, P., Stringer, D., and Wiegand, H.J. (1994) Hepatic Peroxisome Proliferation in Rodents and Its Significance for Humans, *Food Chem. Toxicol. 31*, 857–907.
25. Conway, J.G., Cattley, R.C., Popp, J.A., and Butterworth, B.E. (1989) Possible Mechanisms in Hepatocarcinogenesis by the Peroxisome Proliferator Di(2-ethylhexyl)phthalate, *Drug Metab. Rev. 21*, 65–102.
26. Reddy, J.K., Warren, J.R., Reddy, M.K., and Lalwani, N.D. (1982) Hepatic and Renal Effects of Peroxisome Proliferators: Biological Implications, *Ann. N.Y. Acad. Sci. 386*, 81–110.
27. Gray, R.H., and de la Iglesia, F.A. (1984) Quantitative Microscopy Comparison of Peroxisome Proliferation by the Lipid-Regulating Agent Gemfibrozil in Several Species, *Hepatology 4*, 520–530.
28. Thomassen, M.S., Norseth, J., and Christiansen, E.N. (1985) Long-Term Effects of High-Fat Diets on Peroxisomal ß-Oxidation in Male and Female Rats, *Lipids 20*, 668–674.
29. Kvannes, J., Eikhom, T.S., and Flatmark, T. (1994) The Peroxisomal ß-Oxidation Enzyme System of Rat Heart. Basal Level and Effect of the Peroxisome Proliferator Clofibrate, *Biochim. Biophys. Acta 1201*, 203–216.
30. Flatmark, T., and Christiansen, E. (1993) in *Peroxisomes: Biology and Importance in Toxicology and Medicine* (Gibson, G., and Lake, B., eds.), pp. 247–275, Taylor & Francis, London.
31. Björkhem, I., Blomstrand, S., Glaumann, H., and Strandvik, B. (1985) Unsuccessful Attempts to Induce Peroxisomes in Two Cases of Zellweger Disease by Treatment with Clofibrate, *Pediatr. Res. 19*, 590–593.

[Received December 20, 1994, and in revised form May 5, 1995; Revision accepted May 30, 1995]

Enteral Feeding a Structured Lipid Emulsion Containing Fish Oil Prevents the Fatty Liver of Sepsis

Susan Lanza-Jacoby*, Hope Phetteplace and Robert Tripp

Department of Surgery, Jefferson Medical College, Philadelphia, Pennsylvania 19107

ABSTRACT: Fish oils (FO) have been shown to reduce plasma triglycerides (TG). In this study we evaluated whether enteral feeding with a structured lipid emulsion (SLE) containing FO and medium-chain triglycerides (MCT) would prevent the hypertriglyceridemia and fatty infiltration of the liver that develops during sepsis. For five days, male Lewis rats (275–300 g) were fed intragastrically a nutritionally complete diet containing a SLE or a similar diet with a soybean oil emulsion (SOE) in place of the SLE. On the fifth day, sepsis was induced by intravenously injecting 8×10^7 live *Escherichia coli* colonies/100 g b.w.; 24 h later the control SLE, septic SLE, control SOE, and septic SOE rats were sacrificed. Diet, but not treatment, had a significant effect on serum TG and free fatty acids (FFA). Feeding the SLE reduced the plasma FFA of the control and septic rats by more than 50% in comparison to both control and septic rats fed the SOE. Soleus muscle activity of lipoprotein lipase from the septic SLE rats was 44% higher than the control SLE rats. Soleus muscle from the septic SLE rats also had a twofold greater activity of lipoprotein lipase than the septic SOE rats. TG did not accumulate in the livers of the septic rats fed SLE when compared to the control SLE rats and the rats fed the SOE. Livers from the septic rats fed the SLE had a third of the TG that were present in the livers from the septic rats fed the SOE. The rate of incorporation of [^{14}C]oleate into liver lipids was significantly lower in septic rats fed SLE than in those fed the SOE. TG esterification was 70% lower in the septic rats fed SLE rather than the SOE. Our findings suggest that the SLE with FO and MCT has a role in the prevention of the sepsis-associated fatty liver by reducing the biosynthesis of liver TG.

Lipids 30, 707-712 (1995).

Gram-negative sepsis produces liver injury and dysfunction, which results in alterations in lipid metabolism, leading to the accumulation of liver lipids. Maintaining normal liver function is essential in preventing liver failure and improving survival rates (1,2). Fatty infiltration of the liver developed in both fasted and fed septic rats and was associated with an increase in lipid synthesis (3,4). Lipids accumulated in freshly isolated hepatocytes from septic rats because the synthesis of *de novo* fatty acids and the synthesis of triglycerides (TG) were increased, while secretion rates remain unaltered (5). Earlier studies with *Diplococcus pneumoniae* infection in rabbits (6) and *Streptococcus pneumoniae* infection in rats (7) have also noted that the synthesis of liver lipids was increased.

Numerous studies have shown that fish oils (FO) (ω3 fatty acids) have hypolipidemic effects in normolipidemic subjects (8,9) and in patients with hyperlipidemia (10). The primary lipid-lowering effect of FOs has been attributed to a reduction in TG production by the liver (11–14), which may result from an inhibition of *de novo* fatty acid synthesis (11,15,16), an increase in hepatic fatty acid oxidation (17), a reduction in adipose tissue lipolysis (18), and/or a subsequent decrease in TG synthesis (19,20). There is also the possibility that FOs may contribute to the TG-lowering effect by increasing the removal of circulating TG by stimulating lipoprotein lipase (LPL) activity (21,22).

In a previous study, we reported that septic rats who were adapted to an FO diet for two weeks prior to inducing sepsis and then fasted overnight did not develop fatty liver compared with septic rats adapted to a corn oil diet (23). Total parenteral nutrition with a 10% lipid emulsion with FOs rather than with safflower oil has been shown to improve survival in septic guinea pigs (24). These findings suggested that FOs may have a role in ameliorating the disturbances in lipid metabolism associated with gram-negative sepsis. Lipid emulsions of soybean or safflower oil, which contain long-chain fatty acids (LCFA) of the n-6 series, are routinely used in parenteral and enteral nutritional formulas as part of the treatment for septic and critically ill patients. However, the use of these conventional lipid emulsions with LCFA has been associated with increased prostaglandin production, acute respiratory distress syndrome, reticuloendothelial system blockade, and hepatic steatosis (25). For these reasons, the conventional lipid emulsions may not be appropriate for the septic or hypermetabolic patient.

Lipid emulsions containing both LCFA and medium-chain fatty acids (MCFA) were developed to provide the essential fatty acid requirement and a source of immediate energy. Medium-chain triglycerides (MCT) offer an advantage in that they are cleared and oxidized rapidly. Developing structured lipids provided another advantage of the ability to design TG with mixtures of fatty acids suitable for a specific metabolic

*To whom correspondence should be addressed at Department of Surgery, Jefferson Medical College, 1025 Walnut St., Philadelphia, PA 19107.

Abbreviations: CH, cholesterol; FFA, free fatty acid(s); FO, fish oil; LCT, long-chain triglycerides; LCFA, long-chain fatty acids; LPL, lipoprotein lipase; MCFA, medium-chain fatty acids; MCT, medium-chain triglycerides; SLE, structured lipid emulsion; SOE, soybean oil emulsion; TG, triglyceride(s).

Copyright © 1995 by AOCS Press

need. Recently Teo *et al.* (26) showed that a structured lipid emulsion (SLE) with FO and MCT was more effective than safflower oil emulsion in reducing protein catabolism associated with burn injury in rats. These investigators also have demonstrated that this SLE abated the development of lactic acidosis in endotoxin-treated guinea pigs (27). Accordingly, this study was designed to evaluate whether feeding SLE containing FO and MCT during the septic episode would alter the development of hypertriglyceridemia and fatty liver. To understand the mechanism for the diet effect, the isolated perfused liver was used to evaluate the production and secretion of TG, and the activity of LPL was measured in adipose tissue and soleus muscle to assess the role of these tissues in the removal of TG.

METHODS

Animals. Male Lewis rats (Harlen Sprague-Dawley, Walkerville, MD) weighing between 275–300 g were used in all experiments. The rats were housed in temperature and humidity controlled rooms on a 12 h light–dark (0600–1800 h) schedule. All the rats were allowed free access to a standard rat diet (Purina Rat Chow #5000; Ralston-Purina, St. Louis, MO) for one week prior to the start of the experiment.

Experimental protocol. Two groups of rats were prepared for surgical placement of the intragastric catheter as described in our previous papers (4,5). Rats were infused with 0.09% NaCl at a rate of 0.5 mL/h and allowed free access to the rat diet for the first 24 h after surgical placement of the catheter. On the second day, one group of rats was infused with a liquid diet (Table 1) consisting of a soybean oil emulsion (SOE) (Intralipid; Baxter, Deerfield, IL), and a second group of rats was infused with the same diet, except that the SOE was replaced with an SLE containing 50% MCT, 40% menhaden oil, and 10% canola oil. These diets were as described previously and provided 21% of the calories as fat (4). On the fifth day of feeding, half the rats in each group were made septic by intravenously injecting 8×10^7 live *Escherichia coli*/100 g body weight. Control rats were injected with 0.09% NaCl. Twenty-four hours later the rats were anesthetized by administering ketamine HCl containing acepromazine (Ketaset; Bristal Laboratories, Syracuse, NY) at a dose of 100 mg/kg body weight. Blood, soleus muscle, and adipose tissue were retained for analysis of serum TG, cholesterol (CH), free fatty acids (FFA), liver TG, total CH, and total phospholipids, and activities of LPL in the soleus muscle and epididymal fat pads, as described in a previous report (4). A 120-min isolated *in situ* recirculating liver perfusion was performed on five to six rats in each group (SOE control, SOE septic, SLE control, and SLE septic). The perfusate was collected at 60 and 120 min and analyzed for incorporation of [1-^{14}C]oleic acid into TG, total CH, and total phospholipids. At the end of the 120 min, livers were removed for measuring the incorporation of [1-^{14}C]oleic acid into liver TG, total CH, and total phospholipids. Livers were then frozen at −80°C for subsequent measurement of TG, CH, and phospholipid phosphorus.

TABLE 1
Composition of Liquid Diets

Nutrient	Soybean oil (per L)	Fish oil/MCT structured lipid (per L)
Amino acids (g)	27.2	27.2
50% Dextrose (g)	250	250
Soybean oil emulsion (mL) (Intralipid)[a]	130	—
Structured lipid (mL) Fish oil/MCT	—	130
Electrolytes[b] (g)	11.6	11.6
Trace elements[c] (mg)	12.74	12.74
Vitamins, M.V. 1–12 (mL)	3.0	3.0

[a]Intralipid from Baxter (Deerfield, IL). MCT, medium-chain triglyceride.
[b]Electrolytes in g/L: 5.2 sodium chloride, 1.9 potassium acetate, 1.1 potassium phosphate, 2.2 calcium gluconate, and 1.2 magnesium sulfate.
[c]Trace elements in mg/L: 9.24 zinc chloride, 2.32 cupric chloride, 7.75 manganese sulfate, and 0.1 chromic chloride; M.V., multi-vitamin infusion.

Liver perfusion. The isolated *in situ* liver perfusion was performed according to the method of Exton (28), which we described in a previous paper (29). The perfusate volume of ≈150 mL of Krebs-Ringers bicarbonate buffer (pH 7.4) containing 0.1% glucose (Sigma, St. Louis, MO), 2% (vol/vol) minimum essential medium amino acid solution (50×) without glutamine (Gibco, Grand Island, NY), 1% (vol/vol) of 200 mM L-glutamine (100×) (Gibco), 3% bovine serum albumin (Sigma), and 30% hematocrit using fresh human erythrocytes was passed through a silastic tube and reoxygenated with 95:5 oxygen/carbon dioxide (30).

The liver was washed by perfusing for 20 min at a rate of 11 mL/min with 100 mL of perfusate before the label was added. This perfusate was removed, and the liver was perfused for 120 min with fresh medium containing 20 μCi of 1-^{14}C]oleic acid (50 mCi/mmol) complexed with albumin (fatty acid/albumin ratio, 9:9); the initial concentration was 0.4 mmol. During the initial perfusion and after the first and second hour of perfusion, bile flow and the concentrations of lactate and pyruvate (Sigma kits No. 826 UV and No. 726 UV) were measured. The lactate/pyruvate ratio was used as an estimate of liver viability (31). At the end of the perfusion, the livers were perfused with cold (4°C) 0.9% NaCl for 3 min; perfusate and liver tissue were extracted with chloroform/methanol (2:1, vol/vol) (32) for analysis of lipids and radioactivity. Separation of lipid by thin-layer chromatography and measurement of the incorporation was described previously (4).

Analysis of tissue LPL activities. Total LPL activity in epididymal fat was measured in deoxycholate extracts. Soleus muscle was prepared for analysis of LPL activity by homogenizing in NH_3–HCl (pH 8.1) using a Polytron (Brinkman Instruments, Westbury, NY) with a PT probe at maximum speed for 2 min at 0°C. The extracts were diluted 1:7 in detergent-free buffer, and 150 μL was mixed with 150 μL of glycerol-stabilized triolein substrate emulsion as described by Nilsson-Ehle and Schotz (33). The extracts were then incubated for

2 h at 37°C. Activity is expressed as μmoles FFA/gram wet weight/hour.

Statistical analysis. The data were evaluated by two-way analysis of variance with testing of the interaction of diet and *E. coli* treatment, then testing of hypothesis of diet and treatment as main effects. Specific comparison of group means was made when F values were significant for diet or treatment using the Bonferroni correction for the *t*-test. Analysis was conducted using Systat (Systat Inc., Evanston, IL).

RESULTS

Effect of SLE on serum lipids. The diet had a significant effect on serum TG and FFA (Fig. 1). Serum TG and FFA concentrations were reduced significantly in rats fed the SLE as compared to rats fed the SOE. The serum FFA of the rats fed the SLE were 50% lower than that of the rats receiving the SOE. There was no significant *E. coli* treatment effect with serum TG, CH, or FFA. The significance of F values was as follows: for TG: diet effect, $P \leq 0.05$; treatment effect, nonsignificant; diet × treatment, nonsignificant; for FFA: diet effect, $P \leq 0.000$; treatment effect, nonsignificant; diet × treatment, nonsignificant; for CH: diet effect, nonsignificant; treatment effect, nonsignificant; diet × treatment, nonsignificant.

Effect of SLE on LPL activities. To evaluate the ability of fat tissue and skeletal muscle to utilize TG, the activities of LPL were measured in the epididymal fat pads and soleus muscle. Sepsis had a significant effect on LPL activities in the epididymal fat, but the diet had no effect (Fig. 2). There was a significant diet and *E. coli* treatment effect on LPL activity in the soleus muscle. The decrease in LPL activities in the adipose tissue was evident in the septic rats fed the SOE or the SLE. Feeding the SLE rather than the SOE increased LPL activities significantly (threefold) in the soleus muscle from the septic rats (septic-SLE = 64.1 ± 7.5 μmoles FFA/g; septic-SOE = 20.1 ± 1.6 μmoles FFA/g), and by 49% in the soleus muscle from the control rats (control-SLE = 43.8 ± 4.6. μmoles FFA/g; control-SOE = 29.3 ± 2.3 μmoles FFA/g). LPL activity was depressed in septic rats fed the SOE in comparison to the control rats fed the SOE. In contrast, the SLE led to a 46% elevation of LPL activity in soleus muscle of the septic compared to the control rats. The significance of F values was as follows: for the epididymal fat pad: diet effect, nonsignificant; treatment effect, $P \leq 0.001$, diet × treatment, nonsignificant; for soleus muscle: diet effect, $P \leq 0.05$; treatment effect, ≤ 0.001; diet × treatment, nonsignificant.

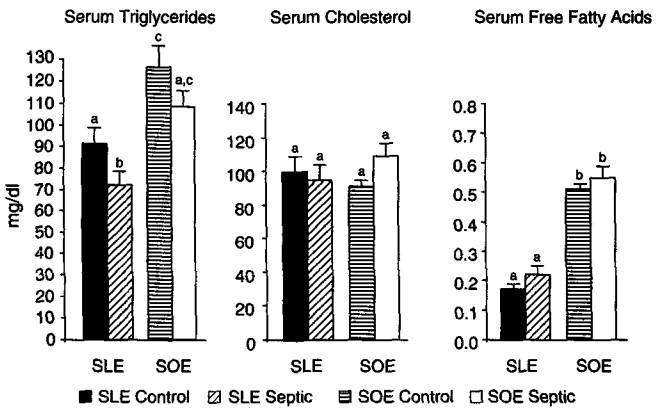


FIG. 1. Concentration of serum triglycerides, cholesterol, and free fatty acids in control and in septic rats fed a structured lipid emulsion (SLE) or a soybean oil emulsion (SOE). Bars represent ± SEM for six rats per group. Values having different superscripts (a,b,c) are significantly different $P \leq 0.05$ by 2 × 2 analysis of variance and Bonferroni correction of *t*-test.

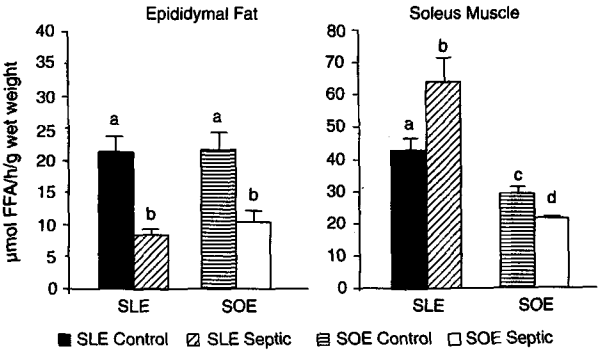


FIG. 2. Lipoprotein lipase activity in the epididymal fat pad and soleus muscle of control and septic rats fed diets containing an SLE or SOE; FFA, free fatty acids. See legend to Figure 1 for details and other abbreviations.

Effect of SLE on the sepsis-induced fatty infiltration of the liver. Table 2 presents the values for the concentrations of TG, total CH, and total phospholipid in the post-perfused livers. Feeding the SLE reduced the TG and total phospholipid contents significantly in livers from both control and septic rats. The livers from the septic rats fed the SOE retained 82% more TG than the livers of the SOE control rats. Feeding the SLE rather the SOE to the septic rats produced a 70% decrease in the content of liver TG.

Effect of SLE on TG production and secretion. The incorporation of [1-^{14}C]oleic acid into liver TG, total CH, and total phospholipids is illustrated in Figure 3. There was a significant diet effect for TG, CH, and total phospholipids. In addi-

TABLE 2
Lipid Contents in Post-Perfuse Rat Liver[a]

Group	Triglycerides (μmol/g)	Cholesterol (μmol/g)	Total phospholipids (μmol Pi/g)
SLE-C	13.2 ± 0.86[b]	15.9 ± 0.8[b]	41.9 ± 1.9[b]
SLE-S	11.3 ± 0.98[b]	13.7 ± 1.4[b]	36.4 ± 1.8[b]
SOE-C	21.1 ± 1.92[c]	18.2 ± 1.9[b,c]	54.4 ± 3.1[d]
SOE-S	38.3 ± 2.72[d]	22.5 ± 1.6[c]	61.8 ± 2.0[d]
Significance of F value			
Diet effect	0.001	0.000	0.000
Treatment effect	0.01	NS	NS
Diet × treatment effect	0.000	NS	NS

[a]Values are means ± SEM of 5–6 rats per group. SLE, structured lipid emulsion; C, control; S, septic; SOE, soybean oil emulsion.
[b–d]Different superscripts are significantly different from each other by 2 × 2 analysis of variance and Bonferroni correction of *t*-test. NS, nonsignificant.

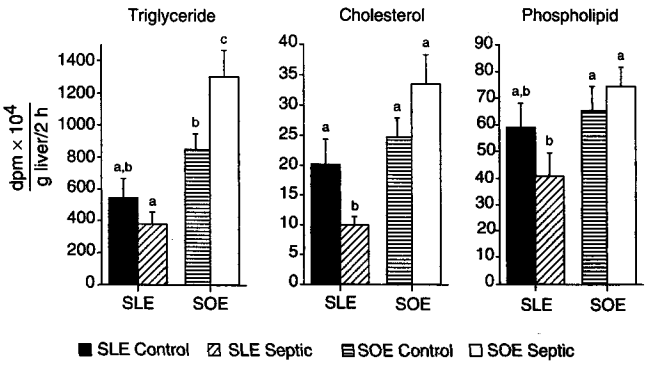


FIG. 3. Rates of incorporation of [1-^{14}C]oleic acid into liver triglycerides, cholesterol, and phospholipids. Livers from control and septic rats fed intragastrically liquid diets with an SLE containing menhaden oil, medium-chain triglycerides, and canola oil or an SOE were perfused as described in the Methods section. See Figure 1 for details and abbreviations.

tion to the reduction in liver TG, TG esterification was also reduced by 71% when septic rats were fed the SLE rather than the SOE. Comparing the control and septic rats fed the SLE, septic rats exhibited a trend toward lower TG and phospholipid esterification, whereas there was a significant reduction in total CH esterification in the livers from the septic rats. Septic rats fed the SOE had a 53% higher rate of TG esterification than that found in the livers from the SOE control rats. The significance of F values was as follows: for TG: diet effect, $P \leq 0.005$; treatment effect, nonsignificant; diet × treatment, nonsignificant; for CH: diet effect, $P \leq 0.000$; treatment effect, nonsignificant; diet × treatment, nonsignificant; for phospholipid: diet effect, $P \leq 0.05$; treatment effect, nonsignificant; diet × treatment, nonsignificant.

The secretion of newly esterified ^{14}C-labeled TG, total CH, and total phospholipids are shown in Figure 4. There was no significant diet or *E. coli* treatment effect for TG or for CH. Total phospholipid secretion was less for the rats fed the SLE rather than for those fed the SOE. The significance of the F values was as follows: for TG: diet effect, nonsignificant; treatment effect, nonsignificant; diet × treatment, nonsignificant; for CH: diet effect, nonsignificant; treatment effect, nonsignificant; diet × treatment, nonsignificant; for phospholipid: diet effect, $P \leq 0.05$; treatment effect, nonsignificant; diet × treatment, nonsignificant.

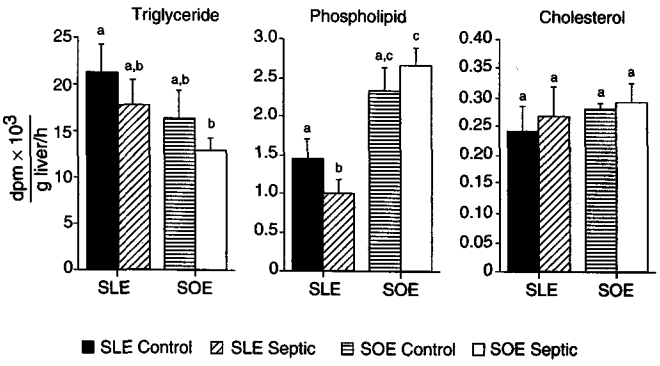


FIG. 4. Rates of incorporation of [1-^{14}C]oleic acid into perfusate triglycerides, cholesterol, and phospholipids. Livers from control and septic rats fed liquid diets with an SLE containing menhaden oil, medium-chain triglycerides, and canola oil or an SOE were perfused as described in the Methods section. See Figure 1 for details and abbreviations.

DISCUSSION

In this study we have shown that an enteral diet containing an SLE with FO and MCT will prevent the development of fatty liver that occurs with sepsis. Lipids accumulate in the liver when there is a disturbance in the balance between synthesis and secretion. Feeding this SLE to the septic rats was associated with a reduction in the synthesis of TG, CH, and phospholipid. Secretion rates were not significantly altered by the diet. The prevention of fatty liver observed with the SLE appears to be due to the decrease in TG synthesis, as the liver content of TG and the incorporation of ^{14}C oleate into TG were both decreased to a similar extent when the septic rats were fed the SLE rather than the SOE. Suppression of TG synthesis and secretion by FOs has been demonstrated in perfused rat livers (11,19,20), cultured rat hepatocytes (13,14), and in HepG2 cells (34). These reports have indicated that the primary mechanism for reduced TG secretion is due to a decrease in TG synthesis. Yet, a recent report by Lang and Davis (35) suggested that decreased secretion may be the primary factor, as they found that TG synthesis was increased in cultured hepatocytes while secretion of TG and apoprotein B were impaired. Otto *et al.* (36) proposed that TG accumulate in the liver when FOs are fed with high-fat diets (30% of calories), but not with lower fat diets (12 and 21% of calories). The *in vitro* experiments conducted by Lang and Davis (35) contained a high concentration of eicosapentaenoic acid or docosahexaenoic acid (1 mM fatty acid). Other reports of *in vivo* studies have also shown that TG was not altered (37) or reduced (11,14,16,36,38) when low- or medium-fat FO diets were fed. In contrast, when high-fat FO diets were fed to rats (39,40), the reduction in plasma lipids was accompanied by a retention in liver TG. The diet used in this study contributed 21% of the calories as fat, which may explain why TG were not retained in the liver. The capacity of the liver to secrete TG may be saturated when there is an excessive supply of exogenous FFA, whereas with low-fat or medium-fat diets, the rate of TG synthesis is the limiting factor for secretion.

The SLE also contained MCT, which are preferentially oxidized rather than esterified into lipids, and are not retained by the liver. There is question as to whether the lipid-lowering effects observed with the SLE are due to the presence of menhaden oil or to MCT. However, one study has shown that lipids accumulated in the livers of septic rats fed either a lipid emulsion with long-chain triglycerides (LCT) or a mixture of LCT and MCT (41). These findings suggest that MCT may not have a role in the prevention of fatty liver observed with the septic rats fed the SLE.

A reduction in availability of FFA in the livers of the SLE-fed rats also may explain the decrease in TG synthesis and, subsequently, the prevention of fatty liver. Serum FFA concentrations were lower in the rats fed the SLE than in the rats fed the SOE, which would lead to a reduced influx of FFA into the liver. Feeding FOs has been shown to reduce blood concentrations of FFA in humans (42), and in rats fed either low- or high-fat diets (14,22). The mechanism for the FO-lowering effect on blood FFA is not fully understood. Parrish *et al.* (18) and Rustan *et al.* (43) have shown that basal lipolysis was lower in epididymal fat from FO-fed rats than in lard-fed rats. Increased activity of LPL in soleus muscle from rats fed the SLE suggests that more fatty acids could be removed from the circulation. An increase in the oxidation of fatty acids by the liver also may reduce fatty acid availability for TG biosynthesis. Studies have shown that mitochondrial and peroxisomal fatty acid oxidation is stimulated in FO-supplemented rats (17,37). The decrease in *de novo* lipogenesis, as was observed by Wong *et al.* (11) and Topping *et al.* (16), also may contribute to the reduced availability of fatty acids for TG biosynthesis.

The hypotriglyceridemic effect of FOs has been demonstrated in animals fed a solid diet (11,14,16,36–40). We also observed a reduction in serum TG with the rats fed the SLE vs. SOE, which may be the result of the higher soleus muscle LPL activity in the rats fed the SLE. Previously we showed that feeding septic rats with the SOE abolished the hypertriglyceridemia of fasted septic rats (4). Our present findings indicate that an SLE with FO and MCT produces similar effects.

Other investigators have shown that skeletal muscle LPL activity was stimulated by feeding FO at 22 and 30% of the calories (21,22), and with prefeeding 10% FO by weight of diet (21% of the calories) then fasting (23). These findings are consistent with our observed increase in the soleus muscle LPL activity in control and in septic rats fed the SLE. The MCFA in the SLE also may influence the clearance of TG. *In vitro* lipid emulsions with MCT are hydrolyzed faster by rat diaphragm LPL than are lipid emulsions with LCFA (44). Yet, *in vitro*, LPL hydrolyzed SLE with MCFA and LCFA slower than physical mixtures of MCT and LCT. The activity of adipose LPL was not altered by feeding the SLE, which was similar to those previously reported (21,36,40).

In this study, we have shown that feeding during the septic episode with SLE containing FO and MCT will prevent the development of fatty liver that occurs when septic rats are fed an SOE. One mechanism for this effect appears to be associated with a reduction in esterification of fatty acids, which could be attributed to a decreased supply of fatty acids presented to the liver. The reduction in serum fatty acids may be related to the increase in skeletal muscle LPL activity. The mechanism for the diet effect on liver and skeletal muscle requires further study. These findings point out that structured lipids with FO and MCT may have an important role in the nutritional support of the septic host.

ACKNOWLEDGMENTS

The authors wish to thank Drs. Vigen K. Babayan and George L. Blackburn for their gift of the structured lipid emulsions. This study was supported by Grant No. GM31828 to S.L.J. from the National Institute of General Medical Sciences

REFERENCES

1. Finley, R.J. (1985) in *Prospectives on Sepsis and Septic Shock*, (Sibbald, W.J., and Sprung, C.L., eds.) pp. 11–26, SCCM, Fullerton.
2. Cerra, F.B., Siegel, J.H., Border, J.R., Wiles, J., and McMenamy, R.R. (1979) *Surgery 86*, 409–422.
3. Wolfe, R.R., Shaw, J.H.F., and Durkot, M.J. (1985) *Am. J. Physiol. 248 (Endocrin. Metab. 11)*, E732–E740.
4. Lanza-Jacoby, S., and Tabares, A. (1990) *Am. J. Physiol. 258*, E676–E685.
5. Lanza-Jacoby, S., and Rosato, E.L. (1994) *Metabolism 43*, 691–696.
6. Gluckian, J.C. (1973) *J. Infect. Dis. 127*, 1–8.
7. Canonico, P.G., Ayala, E., and Rill, W.L. (1977) *Am. J. Clin. Nutr. 30*, 1359–1363.
8. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) *Metabolism 32*, 179–184.
9. Von Lossonczy, T.O., Ruiter, A., Bronsgeest-Schoute, H.C., van Gent, C.M., and Hermus, R.J. (1978) *Am. J. Clin. Nutr. 31*, 1340–1346.
10. Phillipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S., and Illingworth, R.D. (1985) *N. Engl. J. Med. 312*, 1210–1216.
11. Wong, S.H., Nestle, P.J., Timble, R.P., Storer, G.B., Illman, R.J., and Topping, D.L. (1984) *Biochim. Biophys. Acta 72*, 103–109.
12. Nestle, P.J., Connor, W.E., Reardon, M.F., Connor, S., and Boston, R. (1984) *J. Clin. Invest. 74*, 82–89.
13. Nossen, J.O., Rustan, A.L., Gloppestad, S.H., Malbakken, S., and Drevon, G.A. (1986) *Biochim. Biophys. Acta 879*, 56–65.
14. Rustan, A.C., Christiansen, E.N., and Drevon, C.A. (1992) *Biochem. J. 283*, 333–339.
15. Iritani, N., Inoguchi, K., Endo, M., Fukuda, E., and Morita, M. (1980) *Biochim. Biophys. Acta 618*, 378–382.
16. Topping, D.L., Trimble, R.P., and Storer, G.B. (1987) *Biochim. Biophys. Acta 927*, 423–428.
17. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M., and Berge, R.K. (1993) *J. Lipid Res. 34*, 13–22.
18. Parrish, C.C., Pathy, D.A., Parkes, J.G., and Angel, A. (1991) *J. Cell. Physiol. 148*, 493–502.
19. Wong, S.H., and Marsh, J.B. (1988) *Metabolism 37*, 1177–1181.
20. Zhang, Z.J., Wilcox, H.G., Elam, M.B., Castellani, L.W., and Heimberg, M. (1991) *Lipids 26*, 504–511.
21. Herzberg, G.R., and Rogerson, M. (1980) *Lipids 24*, 351–353.
22. Baltzell, J.K., Woolens, J.T., and Otto, D. (1991) *Lipids 26*, 289–294.
23. Lanza-Jacoby, S., Smythe, C., Phetteplace, H., and Tabares, A. (1992) *JPEN 16*, 353–358.
24. Mascioli, E., Leader, L., Flores, E., Trimbo, S., Bistrian, B., and Blackburn, G. (1988) *Lipids 23*, 623–625.
25. Bach, A.C., and Babayan, V.K. (1982) *Am. J. Clin. Nutr. 36*, 950–962.
26. Teo, T.C., DeMichele, S.J., Sellack, K.M., Babayan, V.K., Blackburn, G.K., and Bistrian, B.R. (1989) *Ann. Surg. 210*, 100–107.
27. Teo, T.C., Selleck, K.M., Wan, J.M.F., Pomposelli, J.J., Babayan, V.K., Blackburn, G.L., and Bistrian, B.R. (1991) *Metabolism 40*, 1152–1159.
28. Exton, J.H. (1975) *Methods Enzymol. 39*, 25–37.

29. Tripp, R.J., Tabares, A., Wang H.Z., and Lanza-Jacoby, S. (1993) *J. Surg. Res. 55*, 465–472.
30. Hamilton, R.L., Berry, M.N., Williams, M.C., and Severinhhaus, E.M. (1974) *J. Lipids Res. 15*, 182–187.
31. Mitzkat, H.J., and Meyer, V. (1973) in *Isolated Liver Perfusion and Its Applications*, (Bartosek, I., ed.) pp. 79–86.
32. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1987) *J. Biol. Chem. 226*, 497–509.
33. Nilsson-Ehle, P., and Schotz, M.C. (1976) *J. Lipid Res. 17*, 536–541.
34. Wong, S.H., and Nestel, P.J. (1987) *Atherosclerosis 64*, 139–146.
35. Lang, C.A., and Davis, R.A. (1990) *J. Lipid Res. 31*, 2079–2086.
36. Otto, D.A., Baltzell, J.K., and Wooten, J.T. (1992) *Lipids 27*, 1013–1017.
37. Halminski, M.A., Marsh, J.B., and Harrison, E. (1991) *J. Nutr. 121*, 1554–1561.
38. Yeo, Y.K., and Holub, B.J. (1990) *Lipids 25*, 811–814.
39. Otto, D.A., Tsai, C.E., Baltzell, J.K., and Wooten, J.T. (1991) *Biochim. Biophys. Acta 1082*, 37–48.
40. Haug, A., and Hostmark, A.T. (1987) *J. Nutr. 117*, 1011–1017.
41. Scheig, R. (1968) in *Medium Chain Triglycerides* (Senior, J.R., ed.), University of Pennsylvania Press, Philadelphia, 1968, pp. 39–40.
42. Dagnelie, P.C., Rietveld, T., Swart, R., Stijnen, T., and van den Berg, J.W. (1994) *Lipids 29*, 41–45.
43. Rustan, A.C., Hustvedt, B.E., and Drevon, C.A. (1993) *J. Lipid Res. 34*, 1299–1309.
44. Lutz, O., Lave, T., Frey, A., Meraihi, Z., and Bach, A. (1989) *Metabolism 38*, 507–513.

[Received November 28, 1994, and in final revised form May 8, 1995; Revision accepted May 9, 1995]

Effect of Tumor Necrosis Factor-α on Triglyceride and Phospholipid Content and Fatty Acid Composition of Liver and Carcass in Rats

Nilima Raina, Junichi Matsui, Stephen C. Cunnane and Khursheed N. Jeejeebhoy*

Departments of Medicine and Nutritional Sciences, University of Toronto, Toronto, Ontario M5S 1A8, Canada

ABSTRACT: We have previously shown that a continuous infusion of tumor necrosis factor-α (TNF-α) in rats results in an increase in plasma triglyceride (TG), liver protein and DNA, and at the same time a reduction in muscle protein. However, there is no information on the associated effects of TNF-α on liver and muscle lipids. The present study, therefore, determined the effect of TNF-α on the TG and phospholipid (PL) content and their fatty acid composition, in the liver and carcass of rats and correlated with the plasma levels of insulin, corticosterone, and catecholamaines. Total parenteral nutrition that met the daily nutrient requirements was continuously infused for 10 d through a central vein catheter in two groups of rats, one receiving infused TNF-α (100 μg/kg/d) and a control group. Hepatic TG and PL, expressed either as mg/g dry weight or total organ content, were significantly increased in the TNF-α-infused group compared with controls. Livers of TNF-α infused rats contained significantly less saturated and monounsaturated fatty acids and significantly more polyunsaturated fatty acids (PUFA) of the ω3 and ω6 series than controls. The carcass, on the other hand, contained significantly more monounsaturated and significantly less polyunsaturated fatty acids of the ω6 series than controls. There were no changes in corticosterone level. Although plasma glucose levels were comparable between control and TNF-α infused rats, the latter had a marked increase in insulin levels, demonstrating insulin resistance. In addition TNF-α infused rats had raised norepinephrine levels. The fall in carcass and rise in liver TG and PL are consistent with the presence of insulin resistance and raised norepinephrine levels. However, the increase in PUFA content of the liver, especially the increase in 20:4ω6 levels in liver PL and TG, is likely to be the effect of TNF-α and suggests increased activity of Δ5-desaturase.

Lipids 30, 713–718 (1995).

Sepsis is associated with hypertriglyceridemia (1), which could be primarily due to either an increase in hepatic lipid synthesis (2) or a decrease in lipoprotein lipase (3,4). The disturbance of the host lipid metabolism is thought to be mediated by cytokines (5). The cytokine, tumor necrosis factor-α (TNF-α), is derived from macrophages and has been implicated in the pathogenesis of cachexia as well as derangement of host lipid metabolism (6). A previous study (7) from our laboratory showed that total parenteral nutrition (TPN), providing 14% of total energy by long-chain triglycerides (LCT) as Intralipid 20% (Pharmacia Inc., Mississauga, Ontario, Canada), increased visceral (including liver) protein and DNA content together with wasting of the carcass, despite the same weight gain in control and TNF-α-treated animals. These findings suggested a preferential accumulation of visceral protein at the expense of carcass protein. Raised serum triglyceride (TG) levels in TNF-α-infused rats were also observed by us (7) and others (8–10). However, the effect of TNF-α on the lipid content of carcass and liver in rats receiving TPN has not been reported previously. In addition, these changes have not been related to concomitant changes in hormones which could, in turn, influence lipid metabolism.

We used our previously described rat model (11) to investigate the effect of continuous TNF-α infusion on the total lipids and fatty acid content of both carcass and liver. Two group of rats, TNF-α-infused and controls, were given the same amount of nutrients by TPN and the TG and phospholipid (PL) content of carcass and liver (expressed as mg/g dry weight and total organ content), and their fatty acid composition in carcass and liver (expressed as wt% of total) was determined.

MATERIALS AND METHODS

Animals and treatments. Fourteen male Wistar rats weighing 200–220 g were divided into two groups and housed in individual cages. The animals were kept at an environmental temperature of 22°C in a 12-h light/dark controlled room. They were fed Purina rodent chow (5001) *ad libitum* for a week. Under general anesthesia (sodium pentobarbital: 50 mg/kg i.p.), a silicone catheter was advanced through the internal jugular vein and positioned in the superior vena cava. The catheter was tunneled through the back of the rat and was encircled by a protective wire spring secured to the rat by a stainless-steel button (Insect Labs, Horsham, PA), as described previously (12,13). The rats were individually housed for 6 d post-operatively and given food and water *ad libitum.* Pre-infusion procedure included the keeping of 60-cm long

*To whom correspondence should be addressed at Room #6352, Medical Sciences Building, University of Toronto, Ontario M5S 1A8, Canada.

Abbreviations: FID, flame-ionization detector; LCT, long-chain triglycerides; PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triglycerides; TNF-α, tumor necrosis factor-α; TPN, total parenteral nutrition.

Copyright © 1995 by AOCS Press

catheters patent, by injecting 0.25 mL of heparinized saline (heparin sodium, 10 U/mL) daily at 11:30 a.m. No heparin was given during the actual infusion study. (This protocol was approved by the University of Toronto Animal Care Policies and Guidelines.)

Recombinant human TNF-α, with an endotoxin content of <25 ng lipopolysaccharide/mg TNF-α, was used. The TNF-α was diluted according to the manufacturer's instructions and was stored in 2-mL aliquots, each containing 25 μg of TNF-α, and was kept frozen at −70°C until used. Each aliquot was thawed just before use and was infused intravenously over 24 h in a rat.

The composition of the TPN solution is shown in Table 1. The nutrients met the recommended requirements for rats, and the infused volume was adjusted to 60 mL/d per rat. The amino acid mixture used was Travasol (Baxter, Mississauga, Ontario, Canada). The infused fat was Intralipid 20% (Pharmacia Inc.) containing LCT, which provided 14% of total energy intake (Table 2). Carbohydrate was added to the infusate as 50% dextrose. Vitamins (Multi-1000 containing per 10 mL; vitamin C, 1000 mg; vitamin A, 10,000 I.U.; vitamin D, 1000 I.U.; vitamin E as dl-alpha tocopheryl acetate, 10 I.U.; thiamine as hydrochloride, 45 mg; riboflavin as sodium phosphate, 10 mg; niacinamide, 100 mg; pyridoxine as hydrochloride, 12 mg; d-pantothenic acid as d-panthenol, 26 mg; and benzyl alcohol, 9 mg) and trace elements were also added, in amounts sufficient to meet the daily requirements of the rats.

TABLE 1
Composition of Total Parenteral Nutrition Solution

Dietary sources	Amount
Water	60.0 mL
Dextrose	16.74 g
Amino acids	2.38 g
Fat	1.25 g
Energy	79.4 kcal
Total nitrogen	0.4 g
Nonprotein calories/nitrogen	183.8 kcal
Na	3.64 mmol
Cl	8.71 mmol
Ca	0.376 mmol
Mg	0.317 mmol
Zn	0.000454 mmol
Mn	0.000629 mmol
Cu	0.0008 mmol
Se	0.000015 mmol
Cr	0.0000038 mmol
Acetate	3.715 mmol
Phosphate	0.420 mmol
Iodide	0.000047 mmol
Thiamine	3.75 mg
Riboflavine	0.83 mg
Niacinamide	8.33 mg
Pyridoxine	1.00 mg
Pantothenate	2.17 mg
Vitamin C	83.30 mg
Vitamin A	833.30 IU
Vitamin D	83.30 IU
Vitamin E	0.83 IU

TABLE 2
Composition of Intralipid 20%[a]

Dietary sources	Amount
Soybean oil	20.0 g/100 mL
Egg phosphatide	1.2 g/100 mL
Glycerol	2.5 g/100 mL
Fatty acids	(g/100 g total fatty acids)
16:0	9.2
18:0	2.9
18:1ω9	26.4
18:2ω6	54.3
18:3ω3	7.2

[a]From Pharmacia Inc., Mississauga, Ontario, Canada.

Rats in the control group (n = 7) received a continuous infusion of TPN at the rate of 2.5 mL/h, resulting in the infusion of 60 mL containing 79.4 kcal/24 h (Table 1). The TNF-α-infused group (n = 7) received TPN at the same rate as controls. In addition to TPN, they also received a continuous infusion of TNF-α at the rate of 25 μg/d. The TNF-α was added to the TPN solution, and both were infused together through the same syringe. During the study, the animals were housed individually in metabolic cages, and both catheter and spring were connected to a swivel, allowing them to move freely. The TPN solution was administered continuously for 10 d, at a constant rate, using a Harvard infusion pump (Pump 22; Harvard Apparatus, Wellesly, MA). New TPN solution was mixed aseptically daily in a laminar air flow hood.

Liver and carcass analysis. After completing the 10 d of infusion, the rats were anesthetized with sodium pentobarbital and were killed by exsanguination. The blood was drawn into a heparinized syringe. Plasma was separated and stored at −70°C until biochemical determinations were made. The liver was removed and weighed. The carcass was also weighed. Both liver and carcass were homogenized. An aliquot of each was lyophilized and the dry weight determined. The lipids were extracted as given below and the extracts stored in chloroform under nitrogen at −70°C until further analysis.

Total lipids and fatty acid profiles. Total lipids in liver and carcass were extracted using chloroform/methanol in the ratio of 2:1 (vol/vol) (14), and the extracts were freed from nonlipid material by washing with normal saline. The lipid extracts were then separated by thin-layer chromatography. The plates were developed with hexane/diethyl ether/acetic acid (80:20:2, by vol) to separate TG and PL. The bands were made visible by spraying with 0.02% dichlorofluorescein in methanol and marking the areas under ultraviolet light. The silica from the marked areas was scraped off into glass tubes with Teflon-lined caps.

Methyl esters of the TG and PL fatty acids were prepared by hydrolysis with 2 mL of 14% boron trifluoride in methanol at 90°C for 30 min (15). Although 30 min hydrolysis yields 80.97% recovery of sphingomyelins as against 100% at 90 min hydrolysis (Raina, N., and Jeejeebhoy, K.N., unpublished data), nevertheless, it is not likely to influence the results, be-

cause sphingomyelins, as percentage of total phospholipid, contribute to only 4.2% in rat's liver and 2.7% in rat's skeletal muscle (16).

Fatty acid methyl esters were analyzed on a gas–liquid chromatograph (Hewlett-Packard 5890A; Mississauga, Ontario, Canada) equipped with a fused silica capillary column (Durabond 225/30 mm × 0.2 mm i.d.; Brockville, Ontario, Canada) and a flame-ionization detector (FID). A temperature gradient increasing from 150 to 220°C over 20 min was used. The output of the FID was quantified and recorded on chromatograms (3393 Hewlett-Packard integrator). The total TG and PL were quantitated from peak areas of fatty acids. The peak areas were then referred to areas of the known amounts of 17:0 internal standards derived from added triheptadecanoin and diheptadecanoyl phosphatidylethanolamine. Individual fatty acid compositions of TG and PL in a sample were calculated by multiplying the percentage peak areas of each fatty acid by the concentrations of total TG and PL in that sample. The total values were expressed as concentration (mg/g dry weight) as total organ content. The individual values was expressed as wt% of total lipid.

Statistical methods. The data, presented as mean values ± standard errors of the means, were compared using the Student's unpaired *t*-test. The differences were considered significant at $P < 0.05$.

RESULTS

Liver composition. The liver of TNF-α-infused rats, both as mg/g dry wt and total organ content, contained significantly more TG and PL than controls (Table 3).

TABLE 3
Total Lipids Expressed as Total Organ Content (TOC) and mg/g Dry Weight in the Liver[a] and Carcass[b] of Rats

	Control n = 7	TNF-α n = 7	P[c]
Liver TG			
TOC mg	117 ± 6[d]	200 ± 30	<0.05
mg/g dry weight	34 ± 2	40 ± 3	<0.05
Liver PL			
TOC mg	170 ± 18	387 ± 30	<0.05
mg/g dry weight	48 ± 9	69 ± 9	<0.05
Carcass TG			
TOC g	17 ± 3	15 ± 3	NS
mg/g dry weight	213 ± 31	196 ± 28	NS
Carcass PL			
TOC g	1.1 ± 0.1	0.8 ± 0.1	NS
mg/g dry weight	14.4 ± 0.9	11.1 ± 1.4	NS

[a]The fresh liver weights of control and tumor necrosis factor-α (TNF-α)-treated rats were 12 ± 0.6 and 24 ± 2 g, respectively; TG, triglycerides; PL, phospholipids.
[b]The fresh carcass weights of control and TNF-α-treated rats were 231 ± 5 and 226 ± 4 g, respectively.
[c]$P < 0.05$, indicates significant difference between the TNF-α and the control group. NS, Not significant.
[d]Values are given as mean ± SEM.

Among liver PL fatty acids, only 18:0 was significantly reduced and 22:4ω6 was significantly increased (Table 4). However, the total hepatic PL in TNF-α-infused rats contained significantly less saturated and more polyunsaturates of the ω6 series than did controls.

The liver TG fatty acid composition showed reduction in 16:0 and 18:1ω9 and increases in 18:2ω6, 20:4ω6, 22:4ω6, 22:5ω6, 18:3ω3, 22:5ω6, 22:5ω3, and 22:6ω3 (Table 5). The

TABLE 4
Individual Fatty Acid Composition of Phospholipids in the Carcass and Liver of Rats Expressed as % Weight of Total Lipids

	Liver		Carcass	
Fatty acids	Control	TNF-α	Control	TNF-α
14:0	0.62 ± 0.05	0.54 ± 0.05	1.43 ± 0.08	1.21 ± 0.10
16:0	26.18 ± 1.20	23.66 ± 0.88	22.58 ± 0.21	21.24 ± 0.35[a]
18:0	26.34 ± 1.05	21.47 ± 1.35[a]	14.91 ± 0.92	17.29 ± 0.88
16:1ω7	0.98 ± 0.25	0.97 ± 0.23	2.74 ± 0.26	1.82 ± 0.09[a]
18:1ω9	4.69 ± 0.75	4.90 ± 0.54	12.63 ± 0.20	11.54 ± 0.37
18:2ω6	19.20 ± 1.91	20.59 ± 1.23	22.74 ± 0.93	20.16 ± 0.56
18:3ω3	0.33 ± 0.07	0.18 ± 0.02	0.38 ± 0.06	0.32 ± 0.01
20:3ω6	0.98 ± 0.10	0.64 ± 0.09	0.78 ± 0.03	1.00 ± 0.03[b]
20:4ω6	15.74 ± 1.72	20.16 ± 1.43	10.47 ± 0.33	11.99 ± 0.32[a]
20:5ω3	—	—	0.29 ± 0.10	0.48 ± 0.12
22:4ω6	0.36 ± 0.05	0.90 ± 0.09[b]	0.92 ± 0.04	1.04 ± 0.05
22:5ω6	0.38 ± 0.12	0.43 ± 0.07	0.27 ± 0.01	0.31 ± 0.02
22:5ω3	0.46 ± 0.06	0.51 ± 0.05	1.38 ± 0.28	1.93 ± 0.07
22:6ω3	4.16 ± 0.36	4.94 ± 0.21	8.65 ± 0.47	9.83 ± 0.33
Saturated	53.13 ± 1.81	45.68 ± 1.63[a]	38.92 ± 0.76	39.75 ± 0.89
Monounsaturated	5.67 ± 0.80	5.87 ± 0.43	15.37 ± 0.43	13.36 ± 0.43[a]
Polyunsaturated				
ω6	36.31 ± 1.69	42.72 ± 1.09[a]	35.18 ± 0.73	34.45 ± 0.79
ω3	4.89 ± 0.45	5.73 ± 0.26	10.53 ± 0.48	12.45 ± 0.35[a]

[a]$P < 0.05$. [b]$P < 0.01$ significant difference between the TNF-α and the control group. See Table 3 for abbreviation.

TABLE 5
Individual Fatty Acid Composition of Triglycerides in the Carcass and Liver of Rats Expressed as % Weight of Total Lipids

	Liver		Carcass	
Fatty acids	Control	TNF-α	Control	TNF-α
14:0	1.65 ± 0.14	1.39 ± 0.12	3.76 ± 0.15	4.21 ± 0.15
16:0	30.73 ± 1.61	21.27 ± 1.31[b]	24.01 ± 0.41	24.69 ± 0.62
18:0	5.19 ± 0.80	4.68 ± 0.69	3.48 ± 0.08	3.80 ± 0.25
16:1ω7	3.64 ± 0.50	3.21 ± 0.67	10.25 ± 0.33	11.02 ± 0.55
18:1ω9	21.91 ± 0.91	18.61 ± 0.32[a]	24.72 ± 0.35	25.52 ± 0.45
18:2ω6	32.12 ± 2.68	42.24 ± 2.03[a]	29.63 ± 0.41	26.79 ± 0.81[a]
18:3ω3	0.71 ± 0.12	1.11 ± 0.08[a]	1.72 ± 0.17	1.57 ± 0.10
20:3ω6	0.52 ± 0.15	0.50 ± 0.07	0.17 ± 0.01	0.18 ± 0.03
20:4ω6	2.27 ± 0.20	4.32 ± 0.60[a]	1.18 ± 0.11	1.16 ± 0.06
20:5ω3	0.15 ± 0.02	0.15 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
22:4ω6	0.30 ± 0.06	0.97 ± 0.11[b]	0.17 ± 0.02	0.18 ± 0.01
22:5ω6	0.09 ± 0.03	0.35 ± 0.05[b]	0.01 ± 0.00	0.01 ± 0.00
22:5ω3	0.16 ± 0.02	0.35 ± 0.04[b]	0.24 ± 0.01	0.26 ± 0.01
22:6ω3	0.56 ± 0.08	0.85 ± 0.07[a]	0.61 ± 0.03	0.68 ± 0.04
Saturated	37.57 ± 2.32	27.35 ± 1.54[a]	31.25 ± 0.42	32.70 ± 0.64
Monounsaturated	25.54 ± 1.37	21.82 ± 0.57[a]	34.96 ± 0.49	36.54 ± 0.32[a]
Polyunsaturated				
ω6	35.31 ± 2.91	48.38 ± 1.78[a]	32.46 ± 1.34	28.22 ± 0.84[a]
ω3	1.58 ± 0.23	2.45 ± 0.07[b]	2.62 ± 0.20	2.52 ± 0.10

[a]$P < 0.05$. [b]$P < 0.01$ significant difference between the TNF-α and the control group. See Table 3 for abbreviation.

increased TG in the liver contained significantly less saturated and monounsaturated fatty acids but more polyunsaturated fatty acids (PUFA) of the ω3 and ω6 series.

Carcass composition. The TG and PL content of the carcass of TNF-α-infused rats, calculated either as mg/g dry wt or total organ content, were not significantly different from controls (Table 3).

Among carcass PL fatty acids, 16:0 and 16:1ω7 were significantly reduced, and 20:3ω6 and 20:4ω6 were significantly increased (Table 4). However, the total carcass PL in TNF-α-infused rats contained significantly less monounsaturates and more polyunsaturates of the ω3 series than did controls.

The carcass TG fatty acid composition was only changed by a significant reduction in 18:2ω6 (Table 5). The total TG in the carcass contained significantly more monounsaturated fatty acids but significantly less PUFA of the ω6 series than did controls.

Arachidonic acid and linoleic acid in the carcass and liver of control and TNF-α-treated rats. Total arachidonic acid content in the liver of control rats was 29.6 mg and in the TNF-α-treated rats was 86.8 mg (Table 6). Therefore, there was a net gain of 57.2 mg arachidonic acid content in the liver, and, by similar calculations, there was a net loss of 43.5 mg arachidonic acid content from the carcass of the TNF-α treated rats. A reverse trend in the linoleic acid content was also seen: a net gain of 93.9 mg in the liver with a loss of 1100 mg from the carcass in the TNF-α-treated rats (Table 6).

Plasma glucose, insulin, and catecholamines. Plasma insulin and norepinephrine levels were significantly higher in the TNF-α-infused group as compared with the controls (Table 7). Plasma epinephrine, glucose, and corticosterone

TABLE 6
Arachidonic Acid (AA) and Linoleic Acid (LA) Movement from Carcass to Liver with TNF-α Treatment[a]

	Control			TNF-α		
	mg wt	%FA	mg FA	mg wt	%FA	mg FA
20:4ω6						
Liver PL	170	15.8	26.9	387	20.2	78.2
Liver TG	117	2.3	2.7	200	4.3	8.6
AA content			29.6			86.8
Carcass PL	1,100	10.5	115.5	800	12.0	96.0
Carcass TG	17,000	1.2	204.0	15,000	1.2	180.0
AA content			319.5			276.0
18:2ω6						
Liver PL	170	19.2	32.6	387	20.6	79.7
Liver TG	117	32.1	37.6	200	42.2	84.4
LA content			70.2			164.1
Carcass PL	1,100	22.7	249.7	800	20.2	161.6
Carcass TG	17,000	29.6	5032.0	15,000	26.8	4020.0
LA content			5281.7			4181.6

[a]FA, fatty acid; see Table 3 for other abbreviations.

TABLE 7
Plasma Glucose, Insulin, and Catecholamine Levels in Controls and TNF-α Infused Rats[a]

Plasma hormone	Control	TNF-α	Probability
Glucose mmol/L	6.1 ± 0.45	8.5 ± 1.25	NS
Insulin pmol/L	118.3 ± 20.07	550.8 ± 38.5	<0.05
Corticosterone ng/mL	157.8 ± 33.75	206.8 ± 46.18	NS
Norepinephrine nmol/L	1.73 ± 0.64	4.17 ± 0.21	<0.01
Epinephrine nmol/L	2.66 ± 1.38	3.26 ± 1.19	NS

[a]See Table 3 for abbreviations.

levels were not significantly different between the control and the TNF-α-infused rats.

DISCUSSION

Infusion of TNF-α reproduces some of the metabolic effects of sepsis and injury (10). Among these effects are an increase in circulating TG levels due to reduced lipoprotein lipase activity (17–19), but the effect of TNF-α on carcass and liver lipid composition in animals has not been studied in detail. The infusion of TNF-α results in anorexia and reduced food intake. Therefore changes in tissue lipid profiles observed in animals infused with TNF-α will be confounded by the changes in nutrient intake. In order to avoid the effects of reduced nutrient intake, we used a model in which nutrient intake was kept constant in control and TNF-α infused animals, by giving TPN in quantities sufficient to maintain normal growth (7). In other studies where animals received TNF-α by bolus injection, shock occurred with attendant circulatory changes, and these animals had elevated levels of corticosteroid hormones. We have avoided these additional confounding factors in our model by a continuous infusion of TNF-α which, while resulting in marked metabolic and body compositional changes (7,11), did not result in shock and measured corticosterone levels, an index of stress, were comparable to controls (Table 7). Therefore, in this study the changes observed took place in the fed steady-state kept comparable in control and TNF-α infused animals.

In a previous study (7), we showed that when the nutritional status was kept constant by the administration of TPN, there were reciprocal changes in carcass and visceral protein, with the carcass losing and the viscera gaining protein. The present study, therefore, was undertaken to determine the effect of TNF-α on total lipids and fatty acid composition of TG and PL in the liver and the carcass of rats. The two groups of rats (TNF-α and control) received continuously the same amount of nutrients by TPN, in which the lipids infused provided only 14% of total calories.

Liver composition of saturated, ω9, ω6, and ω3 fatty acids in our control rats (Tables 4 and 5) is comparable to those published by Innis (20) in rats receiving TPN with the same lipid source as in this study. Comparable data for the carcass have not been published, to the best of our knowledge.

The TNF-α-infused rats had markedly increased insulin levels (500%) over controls, but plasma glucose levels were similar; therefore, these rats were very resistant to insulin action. The insulin resistance is likely to be due to suppression of the insulin-mediated phosphorylation of the glucose receptor by TNF-α (21). Insulin resistance and the increased norepinephrine levels will increase lipolysis, resulting in increased output of free fatty acids from the carcass. As explained below, the increased free fatty output from the carcass could supply fatty acids necessary for increased accumulation of liver TG and PL (17 and 43% per gram dry weight, respectively) in TNF-α-infused rats.

Although the PL and TG content of the carcass did not significantly change with the infusion of TNF-α, there was a mean loss of 4% in the proportion of ω6 fatty acids in carcass TG. Since the ω6 PUFA of carcass TG fell from 32% of 17 g to 28% of 15 g (Tables 3 and 5) in the TNF-α-infused animal, this change would amount to a loss from carcass of about 1240 mg of ω6 fatty acids. The corresponding gain in the liver of ω6 fatty acids in TG was from 35% of 117 mg to 48% of 200 mg, which is about 55 mg and, by the same calculation, 101 mg in PL. It is clear that the loss in carcass was of a magnitude which could easily supply the gain in liver. The total arachidonic acid and linoleic acid content (Table 6) showed a net gain in the liver with a net loss from the carcass in the TNF-α-treated rats. However, the gain of total liver 18:2ω6 was not in proportion to the loss of total carcass 18:2ω6. Despite the fatty acids' mobilization from carcass to liver, it is apparent that the loss from the carcass exceeds such a transfer, raising the possibility of selective oxidation of PUFA in the carcass or in the liver to supply the increased energy needs induced by the hypermetabolic state induced by TNF-α infusion.

The TNF-α-infused rats had increased liver PL levels with increased content of unsaturated fatty acids. These changes are likely to be due to the insulin-resistant state caused by TNF-α infusion. In streptozotocin-induced diabetes, it was shown acyl-CoA:1-acylglycerol 3-phosphate acyltransferase and acyl-CoA:1-acylglycerol 3-phosphorylcholine acyltransferase were both markedly increased associated with decreased mitochondrial phospholipase A_2 activity and lysophospholipase activity (22). These changes will result in increased PL synthesis. Since both acyl-CoA:1-acylglycerol 3-phosphate acyltransferase and acyl-CoA:1-acylglycerol 3-phosphorylcholine acyltransferase have increased activity toward unsaturated acyl-CoA donors, the PL synthesized will be rich in unsaturated fatty acids.

The abovementioned effects due to insulin resistance cannot however explain the marked increase 20:4ω6 (90%), 22:4ω6 (300%) levels in liver TG with only a 31% increase in 18:2ω6 levels (Table 5). Similarly, there was a significant increase in 20:4ω6 (28%), 22:4ω6 (250%) in liver PL with no change in 18:2ω6 levels (Table 4). These observations suggest an increase in the activity of Δ5-desaturase, which is contrary to the expected change with insulin resistance. In insulin-resistant subjects, increased insulin levels as seen in TNF-α-infused animals, reduced arachidonic acid and longer chain PUFA levels (23), and increased Δ5-desaturase activity (24,25). Therefore, the increased desaturase activity is likely to be a specific effect of TNF-α. In this context, it is of interest that adipocytes lacking the ability to synthesize arachidonic acid are resistant to the cytotoxic effect of TNF-α (26), indicating that inducing desaturase activity may be essential to the cellular effect of TNF-α.

Finally, increased TG synthesis in the liver may also contribute to hypertriglyceridemia observed by us (7) and others (8–10). Hence, the hypertriglyceridemia seen in sepsis may not be due only to reduced lipoprotein lipase. In addition, the shift of PUFA to the liver may provide substrate in that

organ for the production of inflammatory mediators (27) and may contribute to abnormal liver function, which we have observed in TNF-α-infused animals in an earlier communication (7). Further studies need to be done to resolve these issues.

ACKNOWLEDGMENTS

This work was supported by MRC of Canada grant MT-12238 and in part by a grant from Mead Johnson (Bristol-Myers Squibb Company, Evansville, IN). Recombinant human TNF-α, with an endotoxin content of <25 ng lipopolysaccharide/mg TNF-α, was a gift of the Chiron Corporation (Emeryville, CA).

REFERENCES

1. Feingold, K.R., Soued, M., Adi, S., Staprans, I., Neese, R., Shigenaga, J., Doerrler, W., Moser, A.H., Dinarello, C.A., and Grunfeld, C. (1991) Effect of Interleukin-1 on Lipid Metabolism in the Rat, *Arterioscler. Thromb. 11*, 495–500.
2. Feingold, K.R., Seiro, M.K., Adi, S., Moser, A.H., and Grunfeld, C. (1989) Tumor Necrosis Factor Stimulates Hepatic Lipid Synthesis and Secretion, *Endocrinol. 124*, 2336–2342.
3. Feingold, K.R., Soued, M., Staprans, I., Gavin, L.A., Donahue, M.E., Huang, B.J., Moser, A.H., Gulli, R., and Grunfeld, C. (1989) Effect of Tumor Necrosis Factor (TNF) on Lipid Metabolism in the Diabetic Rat, *J. Clin. Invest. 83*, 1116–1121.
4. Butterwith, S.C., and Griffin, H.D. (1989) The Effects of Macrophage-Derived Cytokines on Lipid Metabolism in Chicken (gallus domesticus) Hepatocytes and Adipocytes, *Comp. Biochem. Physiol. 94*, 721–724.
5. Grunfeld, C., and Feingold, K.R. (1991) Tumor Necrosis Factor: Cytokines, and the Hyperlipidemia of Infection, *Trends Endocrinol. Metab. 2*, 213–219.
6. Beutler, B., and Cerami, A. (1988) Tumor Necrosis, Cachexia, Shock, and Inflammation: a Common Mediator, *Ann. Rev. Biochem. 57*, 505–518.
7. Matsui, J., Cameron, R.G., Kurian, R., Kuo, G.C., and Jeejeebhoy, K.N. (1993) Nutritional, Hepatic and Metabolic Effects of an Infusion of Cachectin/TNF in Rats Receiving TPN, *Gastroenterology 104*, 235–243.
8. Feingold, K.R., and Grunfeld, C. (1987) Tumor Necrosis Factor-Alpha Stimulates Hepatic Lipogenesis in the Rat *in vivo*, *J. Clin. Invest. 80*, 184–190.
9. Krauss, R.M., Grunfeld, C., Doerrler, W.T., and Feingold, K.R. (1990) Tumor Necrosis Factor Acutely Increases Plasma Levels of Very Low Density Lipoproteins of Normal Size and Composition, *Endo. 127*, 1016–1021.
10. Starnes, H.F. Jr., Warren, R.S., Jeevanandem, M., Gabrilove, J.L., Larchian, W., Oettega, H.F., and Brennan, M.F. (1988) Tumor Necrosis Factor and the Acute Metabolic Response to Tissue Injury in Man, *J. Clin. Invest. 82*, 1321–1325.
11. Hoshino, E., Pichard, C., Greenwood, C.E., Kuo, G.C., Cameron, R.G., Kurian, R., Kearns, J.P., Allard, J.P., and Jeejeebhoy, K.N. (1991) Body Composition and Metabolic Rate in Rat During a Continuous Infusion of Cachectin, *Am. J. Physio. 260*, E27–E36.
12. Popp, M.B., and Brennan, M.F. (1981) Long Term Vascular Access in the Rat: Importance of Sepsis, *Am. J. Physiol. 241*, H606–H612.
13. Popp, M.B., Morrison, S.D., and Brennan, M.F. (1982) Growth and Body Composition During Long-term Total Parenteral Nutrition in the Rat, *Am. J. Clin. Nutr. 36*, 1119–1128.
14. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
15. Christie, W.W. (1982) *Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids*, 2nd edn., pp. 17–105, Pergamon Press, New York.
16. White, D.A. (1973) The Phospholipid Composition of Mammalian Tissues, in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N., and Dawson, R.M.C., eds.) Vol. 3, pp. 441–459, Elsevier, Amsterdam.
17. Lanza-Jacoby, S., Lansey, S.C., Cleary, M.P., and Rosato, F.E. (1982) Alterations in Lipogenic Enzymes and Lipoprotein Lipase Activity During Gram-Negative Sepsis in the Rat, *Arch. Surg. 117*, 144–147.
18. Cerami, A., and Rouzer, C.A. (1980) Hypertriglyceridemia Associated with Trypanosoma Brucei Brucei Infection in Rabbits: Role of Defective Triglyceride Removal, *Mol. Biochem. Parasitol. 2*, 31–38.
19. Robin, A.P., Askanazi, J., Greenwood, M.R.C., Carpentier, Y.A., Gump, F.E., and Kinney, J.M. (1981) Lipoprotein Lipase Activity in Surgical Patients: Influence of Trauma and Infection, *Surgery 90*, 401–407.
20. Innis, S.M. (1986) Effect of Total Parenteral Nutrition with Linoleic Acid-Rich Emulsions on Tissue ω6 and ω3 Fatty Acids in the Rat, *Lipids 21*, 132–138.
21. Feinstein, R., Kanety, H., Papa, M.Z., Lunenfeld, B., and Karasik, A. (1993) Tumor Necrosis Factor-α Suppressed Insulin-Induced Tyrosine Phosphorylation of Insulin Receptor, *J. Biol. Chem. 268*, 26055–26058.
22. Dang, A.Q., Faas, F.H., and Carter, W.J. (1984) Effects of Streptozotocin-Induced Diabetes on Phosphoglyceride Metabolism of the Rat Liver, *Lipids 19*, 738–748.
23. Borkman, M., Storlien, L.H., Pan, D.A., Jenkins, A.B., Chisholm, D.J., and Campbell, L.V. (1993) The Relation Between Insulin Sensitivity and the Fatty-Acid Composition of Skeletal-Muscle Phospholipids, *Eng. J. Med. 328*, 238–244.
24. Mimouni, V., and Poisson, J.P. (1992) Altered Desaturase Activities and Fatty Acid Composition in Liver Microsomes of Spontaneously Diabetic Wistar BB Rat, *Biochim. Biophys. Acta. 1123*, 296–302.
25. Boustani, S., Causse, J.E., Descomps, B., Monnier, L., Mendy, F., and Crastes de Paulet, A. (1989) Direct *in vivo* Characterization of Δ5 Desaturase Activity in Humans by Deuterium Labeling: Effect of Insulin, *Metabolism 38*, 315–321.
26. Reid, T., Ramesha, C.S., and Ringold, G.M. (1991) Resistance to Killing by Tumor Necrosis Factor in an Adipocyte Cell Line Caused by a Defect in Arachidonic Acid Biosynthesis, *J. Biol. Chem. 266*, 16580–16586.
27. Grunfeld, C., Adi, S., Soued, M., Moser, A., Feirs, W., and Feingold, K.R. (1990) Search for Mediators of the Lipogenic Effects of Tumor Necrosis Factor: Potential Role for Interleukin 6, *Cancer Res. 50*, 4233–4238.

[Received May 19, 1994, and in revised form May 4, 1995; Revision accepted May 22, 1995]

Phospholipid Molecular Species Composition of Developing Fetal Guinea Pig Brain

Graham C. Burdge* and Anthony D. Postle

Child Health, University of Southampton, Southampton SO16 6YD, United Kingdom

ABSTRACT: Adequate accumulation of polyunsaturated essential fatty acids, in particular docosahexaenoic acid (22:6n-3), into membrane phospholipids is critical for optimal fetal brain development. This process is maximal during the period of rapid neurite outgrowth, neuritogenesis, which precedes the major growth phase, myelination. There is no information about differential changes during gestation to individual brain phospholipid molecular species which contain 22:6n-3. Such details of brain development would be concealed by total fatty acid analysis of isolated phospholipid classes. We have detailed phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species compositions in developing fetal guinea pig brain. Total brain PC concentration increased substantially between 40 and 68 (term) d of gestation, corresponding to myelination, while PE increased in a biphasic manner between 25–35 d, which was coincident with onset of neuritogenesis, and 40–68 d. Fetal brain development was accompanied by complex changes in the concentration of individual phospholipid molecular species. During early gestation (25–40 d) 22:6n-3 was enriched in both PC and PE *sn*-1 16:0 molecular species. However, between 40 d and term there was no further increase in brain PC 22:6n-3 content, while brain PE was significantly enriched in both PE18:1/22:6 and PE18:0/22:6. We hypothesize that accumulation of 22:6n-3 into *sn*-1 18:1 and 18:0 species represents establishment of a 22:6n-3-containing membrane PE pool which may be turned over more slowly than *sn*-1 16:0 species. Identification of specific changes in membrane phospholipids which are associated with defined events in brain development may provide a basis for assigning functional roles to individual molecular species.

Lipids 30, 719–724 (1995).

Optimal brain function is dependent upon adequate accumulation of polyunsaturated essential fatty acids (PUFA), in particular docosahexaenoic acid (22:6n-3), into neural membrane phosphatidylethanolamine (PE) (1). The period of maximal 22:6n-3 accumulation is associated with rapid neurite formation and outgrowth (neuritogenesis) which precedes the major brain growth phase (2). Prenatal deprivation of 22:6n-3 supply to developing brain, due to maternal dietary manipulation, has been demonstrated in several species, including the monkey (3), rat (4), and guinea pig (4). Specific effects of such deficit in 22:6n-3 assimilation upon subsequent brain function have not been demonstrated in these animals. However, there is strong circumstantial evidence which suggests inadequate 22:6n-3 accumulation into human fetal brain may have permanent developmental and functional consequences. Preterm human infants who were fed milk formula showed a substantial deficit in IQ at eight years of age compared to controls (5). One possible explanation is that this milk formula did not contain pre-formed 22:6n-3, resulting in a deficit in 22:6n-3 assimilation into infant brain, while infants who were fed breast milk received adequate 22:6n-3 to support brain development. In addition, there is some evidence which suggests that inadequate accumulation of 22:6n-3, due to maintenance of term infants on milk formula which lacked 22:6n-3, may lead to persistence of moderate neurological abnormalities at nine years of age (6).

The period of maximal 22:6n-3 accumulation and neurite formation in developing fetal brain differs between animal species. In human brain development, incorporation of 22:6n-3 into PE starts at about 16 wk of gestation and continues into early postnatal life (7,8), corresponding to the prolonged period of neurite formation. Assimilation of 22:6n-3 into fetal guinea-pig brain is also initiated early in gestation, between 25 and 35 d of gestation, and is largely complete at term (68 d) (9). This process precedes and overlaps neuritogenesis (10). Accumulation of 22:6n-3 into developing rat brain and neurite formation, however, are both largely confined to the first two weeks after birth (11).

Despite the importance of 22:6n-3 in fetal brain development, detailed temporal analysis of 22:6n-3 accumulation into brain phospholipids during gestation has not been addressed. Analyses of phospholipid total fatty acid composition has been reported for fetal human (7), fetal guinea pig (9), and neonatal and adult rat (11) brains. However, such measurements provide no information about the distribution of fatty acids between different brain membrane phospholipid molecular species. Measurement of fatty acid concentration, expressed as a fraction of total phospholipid fatty acids, may conceal specific effects upon individual molecular species which may be important in overall membrane function.

*To whom correspondence should be addressed at Child Health, Level G, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, United Kingdom

Abbreviations: HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated essential fatty acid(s).

Copyright © 1995 by AOCS Press

Molecular species compositions of brain phospholipid have been previously reported for adult human (12), adult rat (13), and neonatal monkey (3). However, although these studies have provided detailed information about brain composition, limited numbers of subjects have been used in each case. To our knowledge, there has been no report of molecular species analysis of fetal brain tissue at different stages of development. In the present study, we have determined the composition and concentration of intact phospholipid molecular species of developing fetal guinea pig brain. The results of this study show that increasing gestational age is accompanied by differential enrichments in specific phosphatidylcholine (PC) and PE molecular species.

MATERIALS AND METHODS

Materials. High-performance liquid chromatography (HPLC) grade methanol was obtained from Rathburn Ltd. (Walkerburn, Scotland). Chloroform and choline chloride were purchased from Merck Ltd. (Poole, Dorset, United Kingdom). *Rhizopus arrhizus* lipase (E.C. 3.1.1.3) was from Boehringer Mannhein (Lewes, Sussex, United Kingdom). All other reagents were obtained from Sigma (Poole, Dorset, United Kingdom).

Animal procedures. Outbred virgin adult female guinea pigs were fed maintenance diet (FD1; Special Diets Services, Witham, Essex, United Kingdom) *ad libitum* both before mating and throughout pregnancy. This diet contained 3.4% (wt/wt) fat, which was composed of 22.2% saturated fatty acids, 30.9% monounsaturated fatty acids, 22.7% linolenic acid (18:2n-6), 16.3% α-linolenic acid (18:3n-3), and 7.9% PUFA, mainly arachidonic acid (20:4n-6), expressed relative to total esterified fatty acids.

At gestational ages 25, 35, 40, and 68 (term) d, fetal guinea pigs (n = 6 fetuses/gestation age) were delivered by Caesarian section as described previously (14). Fetal brains were removed, weighed, and frozen in liquid nitrogen. Fetal brain tissue was stored at –20°C.

HPLC analysis of phospholipid molecular species. Fetal brain tissue (100 mg) was homogenized in 0.9% (wt/vol) NaCl using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). Fifty nmoles each of PC14:0/14:0 and PE14:0/14:0 were added as internal standards. Lipids were extracted with chloroform/methanol (15), and phospholipid classes purified on disposable aminopropyl BondElut cartridges (16) (Jones Chromatography, Hengoed, Mid Glamorgan, United Kingdom).

The primary aim of this study was to detail developmental changes in patterns of fetal brain phospholipid molecular species composition at several time points in gestation. In order to be able to analyze a reasonable number of samples, we chose not to separate diacyl from alkyl/acyl and alkenyl/acyl species. While this approach resulted in a number of species being co-eluted, useful information about patterns of molecular species changes in developing fetal brain was obtained. Samples were analyzed by a random protocol to avoid any artifactual time-dependent trend in the results. In addition, the mean variation derived from the whole analytical procedure in the concentration of each molecular species was estimated to be about 20%. PC and PE molecular species were resolved by reverse-phase HPLC on a 25 cm × 4.6 mm APEX I 3μ ODS column (Jones Chromatography), thermostatically maintained at 50°C using a mobile phase of methanol/water (930:70, vol/vol), containing 40 mM choline chloride at a flow rate of 0.8 mL min^{-1}. PE18:1 alkenyl and acyl/18:1 co-eluted with PE18:0/22:6 using this solvent system. These molecular species were resolved by analysis of each sample on the same reverse-phase column maintained at 20°C using a mobile phase of methanol/water (975:25, vol/vol) containing 40 mM choline chloride at a flow rate of 1.0 mL min^{-1}. Mass of eluted molecular species was determined by post-column derivatization with 1,6-diphenyl-1,3,5-hexatriene and fluorescence detection (17). Routine confirmation of phospholipid composition of eluted peaks was provided by on-line determination of ultraviolet absorbance at 205 nm, before the post-column formation of the fluorescence derivative (9). Absorbance at 205 nm was a function both of concentration and of degree of acyl unsaturation of the individual molecular species. The ratio of this integrated absorbance response to phospholipid mass, determined from the fluorescence response, was a constant for each individual molecular species determined by degree of acyl unsaturation. In addition to this method for the validation of species compositions during routine analysis, more detailed assignment of species identities was provided by additional analysis of representative samples. Fatty acid composition of eluted peaks from the reverse-phase HPLC resolutions of PC and PE species was determined by hydrolysis and methylation with BF_3 and analysis by gas chromatography. The *sn*-1 alkenyl *sn*-2 acyl molecular species were identified by susceptibility to hydrolysis with HCl (18). Total PE extracts were resuspended in 500 μL chloroform. HCl was added to a final concentration of 0.4M, and samples were incubated with continuous stirring for 2 min at room temperature. Chloroform and methanol were then added to a final ratio of 1:1, and phase separation was achieved by the addition of isotonic saline and water. The chloroform phase was aspirated and dried under N_2 at 40°C. The *sn*-1 alkyl *sn*-2 acyl species were identified by resistance to hydrolysis with *R. arrhizus* lipase following hydrolysis with HCl (19). PE samples were resuspended in phosphate buffered saline (pH 7.4) (100 μL) by vortexing. The 1000U *R. arrhizus* lipase were added, and the mixture incubated for 2 h at 37°C. The reaction was stopped by addition of chloroform/methanol (1:1, vol/vol) followed by saline and water. The chloroform phase was collected and dried under N_2.

Statistical analysis. Statistical analysis was carried out using the Kruskal Wallis test for multiple comparisons of variables.

RESULTS

Fetal brain growth. Measurement of fetal guinea-pig brain weight showed that between 25 and 40 d of gestation, which

TABLE 1
Fetal Guinea Pig Brain Phosphatidylcholine Composition[a]

	Phosphatidylcholine (nmoles/g)			
	Gestational age (d)			
Molecular species	25	35	40	68
1. 16:0/16:1	858 ± 131	722 ± 127	753 ± 112	523 ± 83[b–d]
2. 16:0/22:6	199 ± 43	402 ± 126[b]	905 ± 328[b,c]	1036 ± 93[b,c]
3. 16:0/20:4	633 ± 111	576 ± 179	574 ± 162	692 ± 117
4. 16:0/18:2	415 ± 140	375 ± 72	616 ± 196	960 ± 78[b–d]
5. 16:0/16:0	2430 ± 242	2435 ± 637	3439 ± 975	4398 ± 732[b–d]
6. 16:0/18:1	2879 ± 152	2588 ± 820	2600 ± 464	4287 ± 398[b–d]
7. 18:0/22:6	759 ± 318	516 ± 177	1247 ± 181[b,c]	1647 ± 355[b,c]
8. 18:0/20:4	209 ± 31	597 ± 148[b]	753 ± 84[b]	682 ± 250[b]
9. 18:0/18:2	87 ± 13	95 ± 25	150 ± 43	600 ± 249[b–d]
10. 16:0/18:0	810 ± 186	596 ± 172	1534 ± 246[b,c]	1546 ± 205[b,c]
11. 18:0/18:1	510 ± 70	520 ± 134	546 ± 62	1703 ± 168[b–d]
Total	10278 ± 750	9913 ± 2711	13941 ± 1176[b]	19362 ± 1859[b–d]

[a]Results of high-performance liquid chromatography analysis of phosphatidylcholine molecular species compositions in fetal guinea-pig brain expressed as mean ± SD; n = 6 fetuses/gestational age.
[b–d]Results significantly ($P < 0.05$) different from 25, 35, and 40 d of gestation, respectively. The wet weights of fetal brains were 1.0 ± 0.1 g, day 25; 1.4 ± 0.4 g, day 35; 1.0 ± 0.4 g, day 40, and 2.5 ± 0.6 g[b–d], day 68.

corresponds to the onset of neurite formation (8), there was no significant change in brain mass (Table 1). The increase in brain mass during the major growth phase, between 40 and 68 d of gestation (Table 1), has been previously shown to be related to deposition of myelin (2).

Fetal brain PC composition. Brain total PC concentration (nmoles/g), which indicates amount of cell membrane, increased 1.4-fold between 35 and 40 d and by a further 1.4-fold between 40 and 68 d (Table 1). However, PC mass per brain, which reflects brain growth, did not change significantly between 25 and 40 days of gestation, but increased 3.4-fold between day 40 (13.1 ± 1.1 μmoles/brain) and day 68 (45.2 ± 1.8 μmoles/brain).

Analysis of individual molecular species showed that increasing gestational age was accompanied by complex, specific modifications to brain membrane composition. Eleven PC molecular species were consistently identified which accounted for >92% molecular species present (Table 1, Fig. 1). At all gestation ages, PC16:0/16:0 and PC16:0/18:1 were the major components (Table 1). PC16:0/22:6 and PC18:0/20:4 concentration increased 2.0- and 2.9-fold, respectively, between gestational ages day 25 and day 35 (Table 1). However, due to statistically nonsignificant decreases in other molecular species, there was no change in total PC concentration (nmoles/g) between 25 and 35 d of gestation (Table 1). PC16:0/22:6 and PC18:0/20:4 concentrations also increased 2.3- and 2.4-fold, respectively, between day 35 and day 40. This was accompanied by increased PC18:0/22:6 (2.4-fold) and PC16:0/18:0 (2.6-fold) concentration (Table 1). The concentration of all PC molecular species identified increased between 40 d of gestation and term (68 d), with the exception of PC16:0/16:1, which decreased 1.4-fold (Table 1). The increment in concentration (nmoles/g) for the majority of PC molecular species was the same as the overall increase in PC concentration. However, between day 40 and day 68, the concentration of PC18:0/18:2 and PC18:0/18:1 increased 4.0- and 3.1-fold, respectively, indicating specific enrichment in these molecular species (Table 1).

Fetal brain PE composition. Fetal brain total PE concentration (nmoles/g) increased 1.7-fold between gestational ages day 25 and day 35, and 3.1-fold between day 40 and term (68 d) (Table 2), whereas there was no significant change in PC concentration during this period (Table 1). Total PE concentration per brain increased 2.4-fold between day 25 (3.1 ± 0.6 μmoles/brain) and 35 d of gestation (7.3 ± 0.9 μmoles/brain), and 7.6-fold between day 40 (5.7 ± 1.4

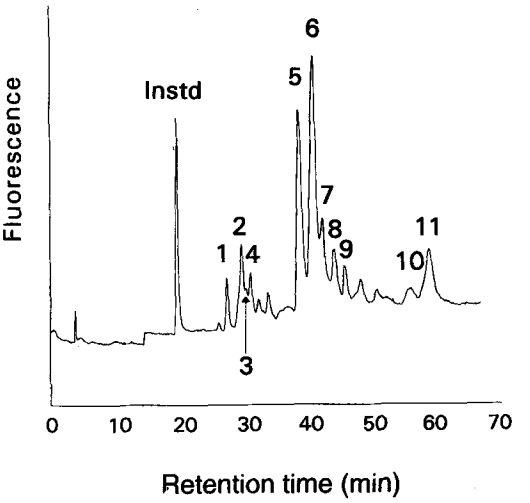


FIG. 1. High-performance liquid chromatography chromatogram of term (68 d) fetal guinea pig brain phosphatidylcholine (PC) molecular species. Instd = PC14:0/14:0 internal standard. Peak numbers correspond to individual molecular species whose identities are indicated in Table 1.

TABLE 2
Fetal Guinea Pig Brain Phosphatidylethanolamine Composition[a]

	Phosphatidylethanolamine (nmoles/g)			
	Gestational age (d)			
Molecular species	25	35	40	68
1. 16:0/22:6	215 ± 117	523 ± 173[b]	565 ± 86[b]	1419 ± 317[b–d]
2. 16:0/20:4	147 ± 47	226 ± 65	242 ± 57[b]	450 ± 44[b–d]
3. 16:0/22:6A	284 ± 93	643 ± 112[b]	605 ± 124[b]	1557 ± 206[b–d]
4. 18:1/22:6	199 ± 55	252 ± 50	298 ± 111	1002 ± 246[b–d]
5. 18:1/20:4A,P	279 ± 94	407 ± 136	396 ± 88	1439 ± 386[b–d]
6. 18:0/22:6[e]	389 ± 68	347 ± 70	542 ± 88[b,c]	2308 ± 345[b–d]
18:1/18:1A,P	155 ± 27	149 ± 28	216 ± 35[b,c]	1519 ± 479[b–d]
7. 18:0/20:4	305 ± 62	1061 ± 187[b]	934 ± 269[b]	2021 ± 394[b–d]
8. 18:0/22:6A	636 ± 146	825 ± 282	869 ± 251	1414 ± 258[b–d]
9. 18:0/22:6P, 18:0/20:4P	277 ± 117	310 ± 108	535 ± 236	1377 ± 144[b–d]
10. 16:0/18:0	185 ± 74	394 ± 179	463 ± 63	554 ± 240[b,c]
Total	3106 ± 561	5339 ± 1039[b]	5749 ± 1451[b]	17524 ± 1748[b–d]

[a]Results of high-performance liquid chromatography (HPLC) analysis of phosphatidylethanolamine (PE) molecular species compositions in fetal guinea-pig brain expressed as mean ± SD; n = 6 fetuses/gestational age. "A" indicates peaks containing a significant amount of *sn*-1 alkyl *sn*-2 acyl PE molecular species. "P" indicates peaks containing a significant amount of *sn*-1 alkenyl *sn*-2 acyl PE molecular species.

[b–d]Results significantly ($P < 0.05$) different from 25, 35, and 40 d of gestation, respectively.

[e]PE16:0/22:6 + PE18:1/18:1A,P appear as a single peak (6) in Figure 2. PE16:0/22:6 and PE18:1/18:1 A,P were separated by HPLC on an ODS 3 μ APEX I column (Jones Chromatography, Hengoed, Mid Glamorgan, United Kingdom) maintained at 20°C, and the mass of each molecular species was determined as described in the Materials and Methods section.

μmoles/brain) and term (43.2 ± 1.4 μmoles/brain). The difference in PE content per brain between day 35 and day 40 was not significant.

In contrast to fetal brain PC, the corresponding PE fraction was composed primarily of molecular species containing PUFA and included species with *sn*-1 acyl, *sn*-1 alkyl-, and *sn*-1 alkenyl-linked fatty acids (Table 2). Eleven molecular species were consistently resolved which accounted for >97% of total PE (Table 2, Fig. 2) and included an unusually high proportion of *sn*-1 alkyl *sn*-2 acyl species (Table 2, Fig. 2B). The increase in brain PE concentration between day 25 and day 35 was accounted for by increased PE16:0/22:6 (2.4-fold), PE 16:0 alkyl/22:6 (2.3-fold), and PE18:0/20:4 (3.5-fold) (Table 2). However, between day 35 and day 40, there were specific increases in PE18:0/22:6 (1.6-fold) and PE18:1 alkenyl/18:1 (1.4-fold) (Table 2). The concentrations of all fetal brain PE molecular species identified increased between 40 d of gestation and term, with the exception of PE16:0/18:0 (Table 2). The increments in PE16:0/22:6 (2.5-fold), PE16:0/20:4 (1.9-fold), PE 16:0 alkyl/22:6 (2.6-fold), PE18:0/20:4 (2.2-fold), PE18:0 alkyl/22:6 (1.6-fold), and PE18:0 alkenyl/22:6 + PE18:0 alkenyl/20:4 (2.6) (Table 2) concentration were all less than that of total brain PE (3.1-fold). However, the increase in PE18:1/22:6 (4.1-fold), PE16:0 alkenyl/20:4 (6.2-fold), PE18:0/22:6 (5.8-fold), and PE18:0 alkenyl/18:1 (7.0-fold) (Table 2) concentration were greater than that of total PE, indicating that these molecular species were enriched in fetal guinea pig brain between day 40 and term.

DISCUSSION

The results of this study show, for the first time, that growth and development of fetal guinea pig brain is associated with specific changes in the concentration of individual PC and PE molecular species. Fetal guinea pig brain PC was composed mainly of saturated and monounsaturated molecular species while the corresponding PE fraction contained primarily *sn*-2 22:6n-3 and 20:4n-6 species. The molecular species distribution of term fetal guinea pig brain PC and PE was strikingly similar, but not identical, to neonatal and adult rat brain PC and PE (13,20), neonatal monkey brain PE (3), and adult human brain (12) composition. This is in marked contrast to rat (19) and guinea pig (7) liver, which differ substantially in phospholipid composition. The similarity of brain phospholipids between these animal species suggests evolutionary convergence and conservation of molecular species composition. In addition, these results suggest that optimal membrane phospholipid molecular species composition is of critical importance to brain function and is maintained largely independent of diet.

In agreement with previous studies (7–11), the present data shows that enrichment of developing brain phospholipids in PUFA occurred during neuritogenesis which precedes the onset of the major brain growth phase, myelination. Increased concentrations of saturated fatty acids were associated primarily with myelination. Measurement of total PE showed a biphasic increase in concentration, the initial increase being associated with the period of maximal neuritogenesis

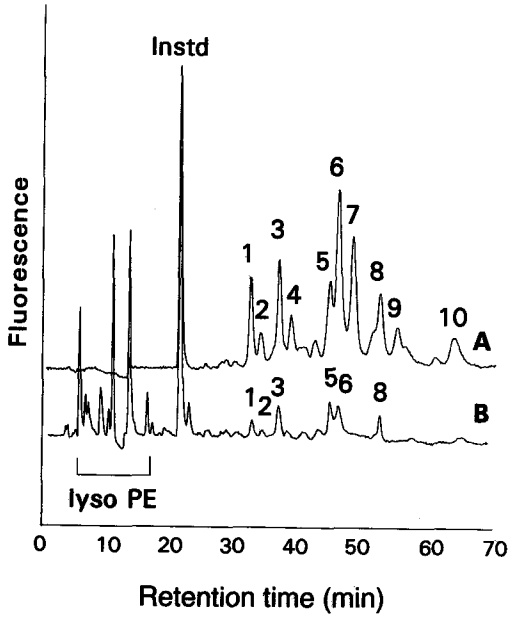


FIG. 2. High-performance liquid chromatography chromatogram of term fetal guinea pig brain phosphatidylethanolamine (PE) molecular species. Numbers correspond to peaks identified in Table 2. A. Intact PE molecular species. B. PE molecular species which were resistant to both digestion with *Rhizopus* lipase and HCl hydrolysis and which were, therefore, *sn*-1 alkyl, *sn*-2 acyl species. Since the PE14:0/14:0 internal standard would have been hydrolyzed by this treatment, it was added after hydrolysis was complete and thus could not be used to correct for losses in recovery. Therefore, PE14:0/14:0 was used to identify peaks according to the retention time relative to this standard. See Figure 1 for other abbreviation.

(d25–d35) while the second phase corresponded with increased total brain mass (d40–d68). Significant increase in PC concentration was restricted to late gestation (d40–d68) and was associated with the period of maximal myelin deposition.

Adequate accumulation of 22:6n-3 and 20:4n-6 is critical for optimal fetal brain growth. Our previous analyses of fetal guinea pig brain PC and PE fatty acid content, expressed as a fraction of total PC or PE fatty acids, showed that 22:6n-3 and 20:4n-6 accumulation into both phospholipid classes was maximal between 25 and 35 d of gestation (9). However, analysis of fetal guinea pig brain PC and PE molecular species showed that increasing gestational age was accompanied by complex changes in the content of individual species, which were not indicated by the previous measurement of total fatty acids (9). For instance, the increased incorporation of 22:6n-3 into brain PC between 25 and 35 d of gestation was primarily into PC16:0/22:6, while 20:4n-6 was accumulated as PC18:0/20:4. Also, the major phase of brain growth and myelination between 40 d of gestation and term was associated with specific enrichment of PC16:0/18:2, PC18:0/18:2, and PC18:0/18:1.

Initial incorporation of 22:6n-3 during the period which corresponded to maximal neurite formation, between 25 and 35 d of gestation (10), was into both PE16:0/22:6 and PE16:0 alkyl/22:6, while accumulation of 22:6n-3 into fetal brain between 35 and 40 d of gestation was primarily into PE18:0/22:6. However, during the major brain growth phase, between 40 d of gestation and term, fetal brain PE was enriched in PE18:1/22:6 and PE18:0/22:6. The *sn*-1 16:0 PE molecular species in both adult rat (21,22) and neonatal guinea pig (14) liver has been shown to be turned over more rapidly than *sn*-1 18:0 PE species. It is, therefore, possible that accumulation of 22:6n-3 into PE16:0/22:6 during neuritogenesis may represent incorporation into molecular species which are turned over rapidly. However, in late gestation when the majority of neurite formation is complete, accumulation of 22:6n-3 into *sn*-1 18:1 and 18:0 PE molecular species may be associated with establishment of a more stable membrane phospholipid pool which is relatively resistant to depletion of PUFA.

It is possible that the contents of more saturated phospholipid species were regulated to compensate for membrane fluidizing effects of increased amounts of unsaturated species. For instance, between 25 and 68 d of gestation, PC16:0/16:0 concentration increased twofold, while that of PE18:1 alkenyl/18:1 increased sevenfold. It is important to recognize, that calculated on a fractional basis, the percentage content of PC16:0/16:0 did not change, while that of PC18:1 alkenyl/18:1 only increased 1.7-fold. This comparison emphasizes that, for future determinations of biological function of individual molecular species of phospholipids, both absolute and fractional concentrations will have to be considered.

The present data shows that fetal guinea pig brain development is accompanied by complex changes in the biologically relevant membrane phospholipid components, which were not detected by analysis of phospholipid fatty acids. These analyses provide an overall pattern of developmental changes in brain phospholipid molecular species composition. Detailed studies on modifications to the molecular species content of developing brain require separation of phospholipid subclasses and are only practical in a relatively limited number of samples. While assignment of specific functions to particular molecular species is not generally possible at present, identification of specific changes in the content of specific species at defined points in development may provide a basis for defining the role of individual membrane phospholipid components. In this context, one useful application of the analytical technique described in this report for the resolution of intact phospholipid species will be to investigate synthesis and turnover of brain phospholipid molecular species from incorporation of radiolabelled precursors.

ACKNOWLEDGMENT

This work was supported by a grant from the Sir Jules Thorn Charitable Trust.

REFERENCES

1. Neuringer, M., Anderson, G.J., and Connor, W.E. (1988) The Essentiality of n-3 Fatty Acids for the Development and Function of the Retina and Brain, *Ann. Rev. Nutr. 8*, 517–541.
2. Dobbing, J., and Sands, J. (1979) Comparative Aspects of the Brain Growth Spurt, *Early Human Dev. 3*, 79–83.

3. Lin, D.S., Connor, W.E., Anderson, G.J., and Neuringer, M. (1990) Effects of Dietary n-3 Fatty Acids on the Phospholipid Molecular Species of Monkey Brain, *J. Neurochem. 55,* 1200–1207.
4. Leat, W.M.F., Curtis, R., Millichamp, N.J., and Cox, R.W. (1986) Retinal Function in Rats and Guinea Pigs Reared on Diets Low in Essential Fatty Acids and Supplemented with Linoleic or Linolenic Acids, *Ann. Nutr. Metab. 30,* 166–174.
5. Lucas, A., Morley, R., Cole, T.J., Lister, G., and Leeson-Payne, C. (1992) Breast Milk and Subsequent Intelligence Quotient in Children Born Preterm, *Lancet 339,* 261–264.
6. Lanting, C.I., Fidler, V., Huisman, M., Towen, B.C.L., and Boersma, E.R. (1994) Neurological Differences Between 9 Year Old Children Fed Breast Milk or Formula Milk as Babies, *Lancet 344,* 1319–1322.
7. Clandinin, M.T., Chappell, J.E., Leong, S., Heim, T., Swyer, P.R., and Chance, G.W. (1980) Intrauterine Fatty Acid Accretion Rates in Human Brain: Implications for Fatty Acid Requirements, *Early Human Dev. 4,* 121–129.
8. Clandinin, M.T., Chappell, J.E., Leong, S., Heim, T., Swyer, P.R., and Chance, G.W. (1980) Extrauterine Fatty Acid Accretion in Infant Brain: Implications for Fatty Acid Requirements, *Early Human Dev. 4,* 131–138.
9. Burdge, G.C., and Postle, A.D. (1994) Hepatic Phospholipid Molecular Species in the Guinea Pig. Adaptations to Pregnancy, *Lipids 29,* 259–264.
10. Burdge, G.C., and Postle, A.D. (1993) Supply and Accumulation of Docosahexaenoic Acid into Developing Fetal Guinea Pig Brain Phosphatidylethanolamine Molecular Species, *Early Human Dev. 32,* 223. [Abstract]
11. Sinclair, A.J., and Crawford, M.A. (1972) The Accumulation of Arachidonate and Docosahexaenoate in the Developing Rat Brain, *J. Neurochem. 19,* 1753–1758.
12. Wilson, R., and Bell, M.V. (1993) Molecular Species Composition of Glycerophospholipids from White Matter of Human Brain, *Lipids 28,* 13–17.
13. Hullin, F., Kim, H.-Y., and Salem, N. (1989) Analysis of Aminophospholipid Molecular Species by High-Performance Liquid Chromatography, *J. Lipid Res. 30,* 1963–1975.
14. Burdge, G.C., Kelly, F.J., and Postle, A.D. (1993) Mechanisms of Hepatic Phosphatidylcholine Synthesis in the Developing Guinea Pig: Contributions of Acyl Remodelling and of *N*-Methylation of Phosphatidylethanolamine, *Biochem. J. 290,* 67–73.
15. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. 37,* 911–923.
16. Caesar, P.A., Wilson, S.J., Normand, I.C.S., and Postle, A.D. (1988) A Comparison of the Specificity of Phosphatidylcholine Synthesis by Human Fetal Lung Maintained in Either Organ or Organotypic Culture, *Biochem. J. 253,* 451–457.
17. Postle, A.D. (1987) Method for the Sensitive Analysis of Individual Molecular Species of Phosphatidylcholine by High-Performance Liquid Chromatography Using Post-Column Fluorescence Detection, *J. Chromatogr. 415,* 41–51.
18. Pugh, E.L., Kates, M., and Hanahan, D.J. (1977) Characterization of the Alkyl Ether Species of Phosphatidylcholine in Bovine Heart, *J. Lipid Res. 18,* 710–716.
19. Tyagi, S.R., Burnham, D.N., and Lambeth, J.D. (1989) On the Biological Occurrence and Regulation of 1-Acyl and 1-*O*-Alkyl-Diradylglycerols in Human Neutrophils. Selective Destruction of Diacyl Species Using *Rhizopus* Lipase, *J. Biol. Chem. 264,* 12977–12982.
20. Leray, C., Sarlieve, L.L., Drefus, H., Massarelli, R., Binaglia, L., and Freysz, L. (1994) Molecular Species of Choline and Ethanolamine Glycerophospholipids in Rat Brain Myelin During Development, *Lipids 29,* 77–81.
21. Burdge, G.C., Hunt, A.N., and Postle, A.D. (1994) Mechanisms of Hepatic Phosphatidylcholine Synthesis in Adult Rat: Effects of Pregnancy, *Biochem. J. 303,* 941–947.
22. Samborski, R.W., Ridgway, N.D., and Vance, D.E. (1990) Evidence that only Newly-Made Phosphatidylethanolamine is Methylated to Phosphatidylcholine and that Phosphatidylethanolamine is not Significantly Deacylated-Reacylated in Rat Hepatocytes, *J. Biol. Chem. 265,* 18322–18329.

[Received January 19, 1995, and in revised form April 20, 1995; Revision accepted May 24, 1995]

Very Long Chain Polyunsaturated Fatty Acids in the Blubber of Ringed Seals (*Phoca hispida* sp.) from Lake Saimaa, Lake Ladoga, the Baltic Sea, and Spitsbergen

Reijo Käkelä [a,c,*], Robert G. Ackman[b] and Heikki Hyvärinen[a]

[a]Department of Biology, University of Joensuu, SF-80101 Joensuu, Finland, [b]Canadian Institute of Fisheries Technology, Technical University of Nova Scotia, Halifax, Nova Scotia, B3J 2X4 Canada and [c]Mekrijärvi Research Station, University of Joensuu, SF-82900 Ilomantsi, Finland

ABSTRACT: Blubbers of four ringed seal subspecies from Lake Saimaa, Lake Ladoga, the Baltic Sea, and Spitsbergen were analyzed for very long chain polyunsaturated fatty acids (VLCPUFA; $>C_{22}$) using gas–liquid chromatography and gas chromatography/mass spectrometry. The VLCPUFA of the blubber oils were mainly n-3 polyunsaturated fatty acids—23:5n-3, 24:3n-3, 24:4n-3, 24:5n-3, 24:6n-3, 26:5n-3, 26:6n-3, and 28:7n-3. The largest VLCPUFA components in all populations were 24:5n-3 (0.1–0.2 wt% of total fatty acids) and 24:6n-3 (0.1%), but 24:4n-3 (0.1%) was also prominent in the Baltic specimens. The blubber oils of the freshwater species contained considerably more 24:4n-6 and 24:5n-6 than the blubbers of the marine species. The differences among the VLCPUFA in these subspecies appear to be mainly due to different dietary VLCPUFA.

Lipids 30, 725–731 (1995).

Very long chain polyunsaturated fatty acids (VLCPUFA; $>C_{22}$) have been detected in aquatic invertebrates (1–4) and in fish (5–7), but no reports on VLCPUFA in marine mammals have been published. Seals deposit large amounts of polyunsaturated fatty acids (PUFA), mainly 22:6n-3, 20:5n-3, and 22:5n-3 (8–10) in their depot fats (blubbers). In addition, the blubbers of freshwater seals are known to contain considerable amounts of n-6 PUFA—20:4n-6, 22:4n-6, and 22:5n-6 (Ref. 10; Käkelä, R., unpublished results). Undoubtedly, diet is the major source of PUFA in seal depots, but it was not known if the VLCPUFA of the diet are incorporated into the blubber in the same way as the shorter chain PUFA. Among the latter, the 22:5n-3 in depot fats of seals and other marine mammals attracts attention, as it is at least one-half of the 22:6n-3 and sometimes equal to it (11), whereas in fish the proportion is normally one-tenth of 22:6n-3. It was felt that the VLCPUFA intermediates could reflect this difference and be of some help in assessing the origin of the VLCPUFA.

*To whom correspondence should be addressed at the Mekrijärvi Research Station, University of Joensuu, Yliopistontie 4, SF-82900 Ilomantsi, Finland.

Abbreviations: GC/MS, gas chromatography/mass spectrometry; PUFA, polyunsaturated fatty acids; VLCPUFA, very long chain polyunsaturated fatty acids.

The biosynthesis of 22:6n-3 and 22:5n-6 has recently been reevaluated (12,13). In rats, 20:5n-3 (in liver) and 20:4n-6 (in seminiferous tubules) are first elongated to 24:5n-3 and 24:4n-6, respectively, then Δ6-desaturated to 24:6n-3 and 24:5n-6 and, finally, chain-shortened to 22:6n-3 and 22:5n-6, respectively. Whether this could also occur in the tissues of marine mammals was not known. Alternatively, the levels and composition of the VLCPUFA of the seals could simply resemble those of their diet. Freshwater fish are known to contain large amounts of n-6 PUFA compared to marine fish (14–18). Accordingly, we have compared the VLCPUFA in the blubber oils of ringed seals from freshwater, brackish water, and ocean populations.

MATERIALS AND METHODS

Blubber samples. The blubbers of the ringed seals from Lake Saimaa (eastern Finland), Lake Ladoga (western Russia), the Bothnian Bay of the Baltic Sea, and Spitsbergen (the Arctic Ocean) were collected as described in Table 1. The blubber samples were stored and transferred frozen, except for those of four individuals from Lake Ladoga. These samples were sent to Joensuu in formalin. Blubber samples cut from the middle of the original sample blocks were homogenized and extracted with hexane using a mechanical blender. After alkali saponification of the recovered oils, the unsaponifiables were removed with diethyl ether, and the fatty acids were liberated with dilute H_2SO_4. The fatty acids were taken up in hexane, and their purity was improved by a thin-layer chromatography clean-up step.

Fractionation, hydrogenation, and methylation of the fatty acids. For qualitative studies, parts of the fatty acid samples were fractionated by urea complexing (19). The free fatty acids were dissolved in an ethanolic solution of urea by refluxing. Fractions were collected during two stages of cooling and filtration, first at room temperature and then in an ice bath. After addition of water and acidification, the fatty acids of nonurea-complexing fractions were extracted into hexane (19). However, only the original fatty acid samples were used for quantitation.

Copyright © 1995 by AOCS Press

TABLE 1
The Ringed Seals (*Phoca hispida* sp.) Studied

Subspecies/origin	n	Cause of death (yr)	Males/females	Weight (kg)	Age
P. hispida saimensis Lake Saimaa, eastern Finland	5	Fishing net (1991–1993)	2/3	20–44	3 mon–2 yr
P. hispida ladogensis Lake Ladoga, western Russia	5	Fishing net (1991–1992)	not known	20–46	4 mon–4 yr
P. hispida botnica Bay of Bothnia, Baltic Sea	5	Shot (1992)	2/3	49–91	2–26 yr
P. hispida hispida Kongsfjorden, Spitsbergen, Arctic Ocean	5	Shot 1992	4/1	48–73	2–6 yr

To detect the proportions of the fatty acids of different chain lengths, samples of total fatty acids, and the nonurea-complexing fractions from urea complexation were hydrogenated. Fatty acids (5 mg) in methanol (30 mL) were stirred with platinum oxide catalyst (1 mg) under hydrogen for one hour. The solution was filtered and the filtrate was concentrated in a nitrogen stream. The hydrogenated fatty acids were recovered in hexane, washed with water, and concentrated under nitrogen.

Before gas–liquid chromatographic analysis, the free fatty acids were converted to methyl esters by heating at 100°C with 7% BF_3 in methanol plus hexane as cosolvent, in glass tubes with Teflon-lined caps for one hour under nitrogen. The esters were extracted into hexane, and the extract was dried and concentrated.

Gas–liquid chromatography. The fatty acid methyl esters were analyzed with a Perkin-Elmer 8420 gas chromatograph (Norwalk, CT) with flame-ionization detection on an Omegawax-320 capillary column (30 m × 0.32 mm i.d., film thickness 0.25 μm; Supelco Inc., Bellefonte, PA). Helium carrier gas was set at 12 psig. The injector and detector temperatures were 250 and 270°C, respectively. Both temperature-programmed (180°C/8 min, 3°C/min, 230°C to end) and isothermal (210 and 245°C) analyses were conducted. The peak areas were recorded on a Perkin-Elmer GP–100 graphic printer. The areas of the PUFA present in sufficiently large amounts were converted to weight percent using theoretical correction factors (20). The good reproducibility of the VLCPUFA analysis and the small deviations of that data from the whole blubber analyses showed that, in spite of small percentages, the accuracy of the quantitation was not significantly reduced.

Gas chromatography/mass spectrometry (GC/MS). The primary identifications of VLCPUFA were made using conventional techniques—the linear log plot procedures based on retention time (21), the occurrence of the components in different urea-complexing fractions, and chain-length studies after hydrogenation. The identifications were confirmed by electron impact MS [Hewlett Packard 5971 mass selective detector on a Hewlett Packard 5890 A gas chromatograph (Avondale, PA) equipped with an OV-1 methyl silicone capillary column (20 m × 0.32 mm i.d., film thickness 0.25 μm), isothermal and temperature programmed runs] after the method of Fellenberg *et al.* (22). These authors have discovered that the intensity of the fragment *m/z* 108 clearly exceeds that of the *m/z* 150 for n-3 PUFA, but *m/z* 150 is more considerable for n-6 PUFA.

Statistics. The fatty acid data (% w/w) were analyzed by one-way analyses of variance, and the means were compared by the Newman Keuls test (Statgraphics 5.0 software; STSC, Inc.). Prior to this analysis, the fatty acid percentages were normalized through arcsine-transformation (23).

RESULTS

The VLCPUFA in the blubber oils (hexane extract) of the ringed seals were mainly n-3 PUFA (Table 2). In GC/MS they produced intense fragments *m/z* 79 and 91, and the fragment *m/z* 108 was considerable, whereas *m/z* 150 was almost absent. The most important VLCPUFA in each subspecies were 24:5n-3 (0.10–0.24 wt%) and 24:6n-3 (0.06–0.13%), the ringed seal of Lake Ladoga having the highest amounts. The blubbers of the Lake Ladoga (Fig. 1) and Spitsbergen seals contained an amount of 24:5n-3 that was twice as high as 24:6n-3, but in the Lake Saimaa and the Baltic specimens these acids were almost equal. In the Baltic ringed seal, 24:4n-3 (0.11%) was also prominent, thus differing from the other subspecies (Fig. 2). The levels of 24:3n-3 and 23:5n-3 were low (0.02% or less) in each subspecies (Figs. 1 and 2), and traces of 25:5n-3 were also seen in some Baltic samples when examined by GC/MS.

Two hexacosapolyenoic acids, 26:5n-3 and 26:6n-3, were found (Fig. 2). The percentages for the ringed seals from Lake Ladoga and the Baltic were 0.02–0.04%, but the Lake Saimaa and Spitsbergen seals had even lower amounts (0.01% or less). No unambiguous interdependence between the n-3 ho-

TABLE 2

Very Long Chain Polyunsaturated Fatty Acid and Their Potential Precursors (wt%, mean ± SE) in the Blubbers of the Ringed Seals from Lake Saimaa (*Phoca hispida saimensis*), Lake Ladoga (*P. hispida ladogensis*), Bothnian Bay (*P. hispida botnica*), and Spitsbergen (*P. hispida hispida*)

Fatty acid	Freshwater		Marine		
	P. hispida saimensis n = 5	*P. hispida ladogensis* n = 5	*P. hispida botnica* n = 5	*P. hispida hispida* n = 5	*P*[a]
20:3n-3	0.41 ± 0.08 AB	0.61 ± 0.05 A	0.35 ± 0.08 B	0.11 ± 0.02 C	.0001
20:4n-3	1.0 ± 0.11 A	1.0 ± 0.08 A	1.1 ± 0.18 A	0.5 ± 0.04 B	.0082
20:5n-3	6.4 ± 0.19 A	7.5 ± 0.34 B	8.1 ± 0.46 BC	8.9 ± 0.43 C	.0015
21:5n-3	0.3 ± 0.01 A	0.4 ± 0.02 A	0.4 ± 0.04 A	0.5 ± 0.02 B	.0001
22:3n-3	0.07 ± 0.02 B	0.16 ± 0.01 A	0.14 ± 0.02 A	0.01 ± 0.003 C	<.0001
22:4n-3	0.2 ± 0.03 AB	0.3 ± 0.02 B	0.5 ± 0.13 B	0.1 ± 0.03 A	.0093
22:5n-3	6.8 ± 0.30	6.3 ± 0.28	5.8 ± 0.43	6.6 ± 0.16	.1466
22:6n-3	9.8 ± 0.18 A	12.3 ± 0.31 B	14.0 ± 0.80 B	13.7 ± 0.52 B	.0001
23:5n-3	0.008 ± 0.001 A	0.018 ± 0.002 B	0.016 ± 0.001B	0.008 ± 0.001 A	.0002
24:3n-3	0.009 ± 0.002 A	0.023 ± 0.005 B	0.023 ± 0.005B	0.006 ± 0.001 A	.0019
24:4n-3	0.023 ± 0.005 A	0.066 ± 0.012 B	0.112 ± 0.022 C	0.012 ± 0.002 A	<.0001
24:5n-3	0.104 ± 0.009 A	0.238 ± 0.013 B	0.096 ± 0.010 A	0.122 ± 0.012 A	<.0001
24:6n-3	0.085 ± 0.014 A	0.125 ± 0.015 B	0.079 ± 0.007 A	0.061 ± 0.004 A	.0052
26:5n-3	0.006 ± 0.001 A	0.030 ± 0.006 B	0.022 ± 0.004 B	0.011 ± 0.002 A	.0002
26:6n-3	0.009 ± 0.003 A	0.026 ± 0.004 B	0.035 ± 0.009 B	0.005 ± 0.001 A	.0003
28:7n-3	trace[c]	0.017 ± 0.004 A[b]	0.049 ± 0.009 B	0.020 ± 0.004 A	.0163
20:4n-6	3.5 ± 0.15 A	1.8 ± 0.06 B	0.8 ± 0.12 C	0.4 ± 0.03 D	<.0001
22:4n-6	0.9 ± 0.07 A	0.4 ± 0.02 B	0.2 ± 0.03 C	0.1 ± 0.02 C	<.0001
22:5n-6	1.2 ± 0.08 A	1.1 ± 0.07 A	0.4 ± 0.08 B	0.1 ± 0.01 C	<.0001
24:4n-6	0.024 ± 0.003 A	0.018 ± 0.002 A	0.006 ± 0.001 B	0.002 ± 0.001 C	<.0001
24:5n-6	0.019 ± 0.002 A	0.019 ± 0.003 A	0.005 ± 0.001 B	0.002 ± 0.000 C	<.0001

[a]One-way analysis of variance. The means are compared by the Newman-Keuls test; values with no common capital letter differ at $P < 0.05$ (the accurate figures for 20:3n-3 and 22:3n-3 support statistics).
[b]n = 3.
[c]Trace amount, <0.001 wt%.

mologues from eicosapolyenoic to hexacosapolyenoic fatty acids was found in the comparison of the subspecies (Table 2). The possible octacosapolyenoic homologues of 26:5n-3 and 26:6n-3 were too small to be detected. However, 28:7n-3 was found (Fig. 2). Although the lipids from the other subspecies contained 0.02–0.05% of 28:7n-3, only trace amounts of this fatty acid were observed in the gas–liquid chromatography analyses of lipids from seals of Lake Saimaa.

The blubbers of the freshwater seals contained two n-6 VLCPUFA, 24:4n-6 and 24:5n-6, both subspecies having approximately 0.02% (Table 2, Fig. 1). These fatty acids were present in negligible proportions in the marine ringed seals. Thus, the contents of 24:4n-6 and 24:5n-6 clearly reflected those of the shorter chain homologues in the blubbers of the seals. The identification of n-6 VLCPUFA was based on retention times and occurrence in fractionated samples, despite their coincidence (24) with much larger n-3 VLCPUFA on the OV-1 column used in GC/MS. Unfortunately, the accuracy of the quantitation of 24:4n-6 was diminished by a plasticizer, dioctyl (di-2-ethylhexyl) phthalate (25), eluting just before it and causing inaccuracies in the integration of the peak area.

The analyses of the hydrogenated fatty acids gave the proportions of the different chain lengths in the fatty acids of the blubbers (Table 3, Fig. 3). The results for fatty acids $>C_{20}$ paralleled those of the PUFA of the unhydrogenated samples, but gave a little larger total for each chain length. When the amounts of 24:1n-9 and 24:0 were added to the sum of tetracosapolyenoic acids, the agreement with hydrogenated 24:0 was satisfactory. In addition, traces of 26:0, 26:1, 28:0, and 28:1 could be seen in the chromatograms of the nonhydrogenated fatty acids, and these contributed to the sums of hexacosanoic and octacosanoic acids. However, these fatty acids failed to totally account for the differences observed, suggesting that there were probably several trace polyunsaturated components that could not be detected individually.

DISCUSSION

Although the VLCPUFA found in the blubbers of the ringed seal subspecies studied were qualitatively the same, clear differences in the abundance of several VLCPUFA were detected (Table 2). The Baltic ringed seal had high proportions of 24:4n-3 and 28:7n-3 compared to other subspecies, and 24:5n-3 was particularly high in the Lake Ladoga specimens. As anticipated, the blubbers of the freshwater seals contained more n-6 VLCPUFA than those of the marine individuals. The proportion of total VLCPUFA in the blubbers also varied; the ringed seal of Lake Ladoga had the largest amount (0.58%), followed by the Baltic (0.44%), Lake Saimaa

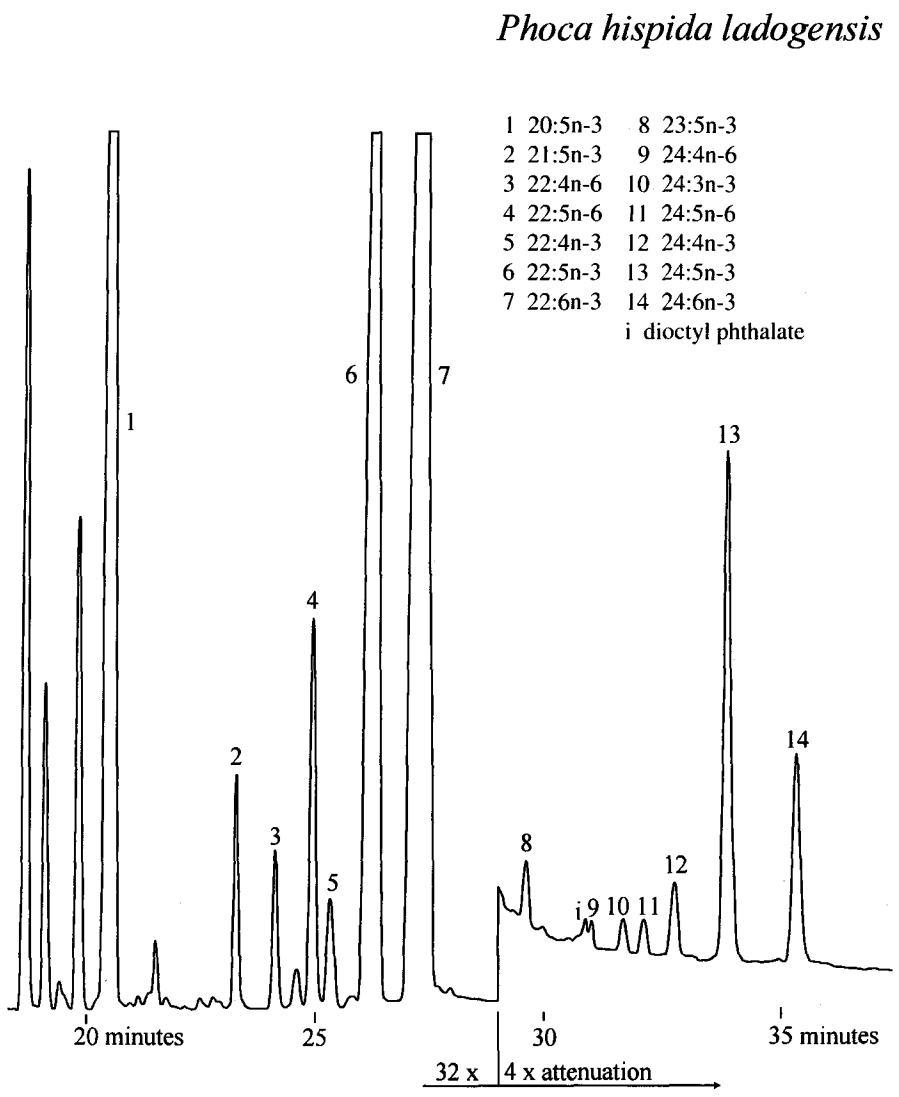


FIG. 1. Partial gas–liquid chromatogram of the total fatty acids from the blubber of the ringed seal of Lake Ladoga showing the area of C_{22-24} PUFA (180°C/8 min, 3°C/min, 230°C to end, Omegawax-320 capillary column; Supelco, Bellefonte, PA).

(0.29%), and Spitsbergen (0.25%) seals. These differences seem to be too large to be due to metabolic divergence only and suggest a dietary explanation.

It is known that the fatty acid composition of seal blubber reflects the dietary fatty acids to a great extent. Especially, the freshwater and marine seals have very different compositions, the former having more of the n-6 PUFA that are abundant in freshwater fish (14–18), and less 20:1n-9 and 22:1n-11 derived from copepoda (10,26). The n-6 PUFA contents of freshwater fish are almost five times those of marine fish, a ratio also found in the long-chain PUFA and VLCPUFA of blubbers from lacustrine and marine seals (Table 2). As far as we know, freshwater fish have not been analyzed for VLCPUFA, and analyses of marine fish for these fatty acids are few. The Baltic herring (*Clupea harengus membras*), an important prey species for the Baltic ringed seal, has been found to contain VLCPUFA of 24–32 carbons in the chain, but the percentages of the total fatty acids have been reported only for 24:4n-3, 24:5n-3, 24:6n-3, 26:5n-3, 26:6n-3, and 28:7n-6 (5). The compositions of the VLCPUFA in the total flesh lipids of the Baltic herring and the ringed seal blubber differ in two ways (Table 4). First, the levels of VLCPUFA in the blubber oils were only one-tenth or less of the herring lipid levels. Second, the 28:7 of the ringed seal was identified to be a n-3 isomer and that of the herring is reported as a n-6 isomer (5). Řezanka (6,27) has used herring (*C. harengus*, unknown origin) as a source of VLCPUFA and, although reporting 28:7n-3 as the main isomer, he also found 28:7n-6 (Table 4). Recently Ota *et al.* (7) found as much as 8 mol% of 24:6n-3 in the flesh triglycerides of flathead flounders from the Sea of Japan.

Could endogenous metabolism (from shorter chain PUFA obtained in the diet) produce VLCPUFA in the seal tissues? Voss *et al.* (28) and Aveldaño *et al.* (13) have shown that

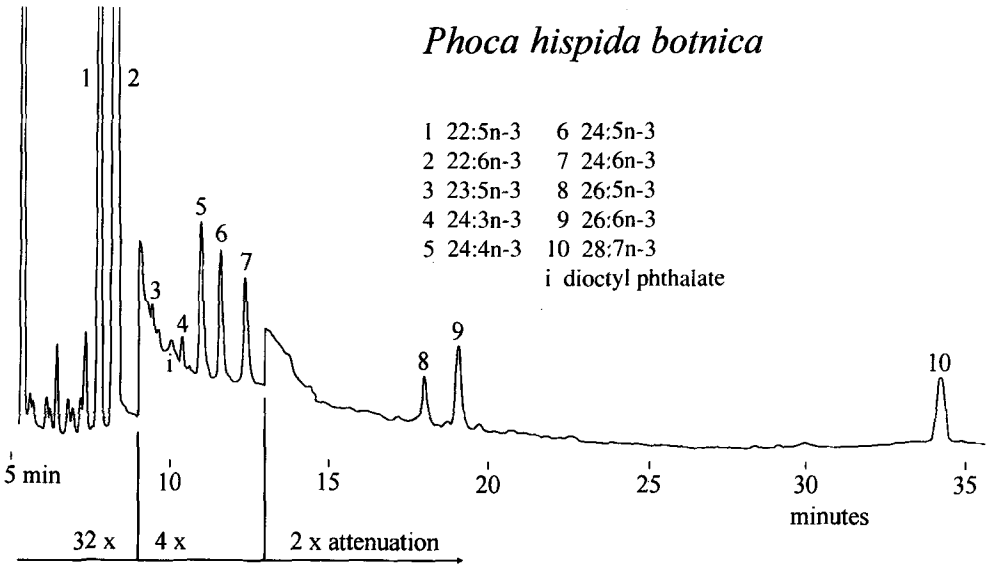


FIG. 2. Partial gas–liquid chromatogram of the total fatty acids from the blubber of the Baltic ringed seal (isothermal 245°C, Omegawax-320 capillary column; Supelco, Bellefonte, PA).

24:5n-3, 24:6n-3, 24:4n-6, and 24:5n-6 are intermediates in the metabolism of 22:6n-3 and 22:5n-6 in the rat, but radiolabel experiments are needed to find out if, or on what scale, these processes occur in seal liver or blubber. Whatever the organisms in which the VLCPUFA of the ringed seal blubbers have been biosynthesized, the biochemistry of these fatty acids seems to follow the pathways proposed in the rat studies. Theoretically, all the VLCPUFA of the ringed seal blubbers could be produced by chain elongation, β- and α-oxidation, Δ5-desaturase and Δ6-desaturase. Even the 28:7n-3 could be seen as being derived from the Δ5-desaturation and subsequent elongation of 26:6n-3.

The occurrence of odd-chain VLCPUFA, 23:5n-3, and trace 25:5n-3 suggest either elongation of 21:5n-3 or α-oxidation. Aveldaño *et al.* (13) have proposed that α-oxidation is an important mechanism in the shortening of fatty acids and that this produces odd-chain PUFA as metabolic intermediates. However, 21:5n-3 was first identified in seal oils (29), where it is more obvious than in fish oils. Possibly the retroconversion of VLCPUFA by β-oxidation is not totally specific, and α-oxidation takes place instead.

Although our knowledge of the biochemistry of PUFA and VLCPUFA has recently expanded, the biological importance of VLCPUFA is not yet understood (30). VLCPUFA have been detected in large amounts in mammalian spermatozoa/testes (1,31–33), brain (34,35), and retina (36–39), which suggests essentiality for at least some specific membrane functions. The tissues known to be rich in VLCPUFA in terrestrial mammals, and also worth analyzing, are those of diving mammals. These obtain large amounts of PUFA from their diets and have special requirements in sensory physiology.

TABLE 3
Hydrogenated Fatty Acids (wt%) of Longer Chain Lengths from a Blubber Sample from the Ringed Seals from Lake Saimaa (*Phoca hispida saimensis*), Lake Ladoga (*P. hispida ladogensis*), Bothnian Bay (*P. hispida botnica*), and Spitsbergen (*P. hispida hispida*)

	Freshwater		Marine	
Fatty acid	*P. hispida saimensis*	*P. hispida ladogensis*	*P. hispida botnica*	*P. hispida hispida*
20:0	13.78	12.77	12.67	19.96
21:0	0.41	0.61	0.78	0.71
22:0	20.21	24.59	25.18	22.35
23:0	0.05	0.07	0.04	0.05
24:0	0.42	0.73	0.49	0.34
25:0	trace	trace	0.01	0.01
26:0	0.02	0.11	0.10	0.03
27:0	ND[a]	ND	trace	ND
28:0	trace[b]	0.02	0.04	0.02

[a]Not detected. [b]Trace amount, <0.005 wt%.

TABLE 4
Comparison of the Main Very Long Chain Polyunsaturated Fatty Acids (VLCPUFA) (wt%) of the Blubber from the Baltic Ringed Seal (*Phoca hispida botnica*) with the VLCPUFA of the Baltic Herring (*Clupea harengus membras*) Flesh and the VLCPUFA of a Fraction of Silver-Ion Exchange High-Performance Liquid Chromatography (HPLC) from Herring (*C. harengus*, unknown origin)

Fatty acid	Baltic ringed seal	Baltic herring[a]	Herring, HPLC fraction[b]
24:4n-3	0.11	1.3	5.65
24:5n-3	0.10	1.4	6.09
24:6n-3	0.08	0.9	3.91
26:5n-3	0.02	0.5	2.16
26:6n-3	0.04	0.7	3.04
28:7n-3	0.05	—	1.97
28:7n-6	—	0.4	0.43

[a]From Reference 5. [b]From Reference 6.

ACKNOWLEDGMENTS

We are grateful to Drs. E. Helle (Finnish Game and Fisheries Research Institute, Helsinki), C. Lydersen (University of Oslo), and M.O. Hammill (Institut Maurice Lamontagne, Quebec) for donating the Baltic and Spitsbergen blubbers. Dr. O. Lavrantieva (Goshniorsk, St. Petersburg) and Ph. Lic T. Sipilä provided us with the Lake Ladoga samples. Dr. R. Julkunen-Tiitto assisted in GC/MS. This study was supported by a grant (to R.K. and H.H.) from the Academy of Finland.

REFERENCES

1. Řezanka, T. (1989) Very-Long Chain Fatty Acids from the Animal and Plant Kingdoms, *Prog. Lipid Res. 28*, 147–187.
2. Vysotskii, M.V., and Svetashev, V.I. (1991) Identification, Isolation and Characterization of Tetracosapolyenoic Acids in Lipids of Marine Coelenterates, *Biochim. Biophys. Acta 1083*, 161–165.
3. Dembitsky, V.M., Řezanka, T., and Kashin, A.G. (1993) Comparative Study of the Endemic Freshwater Fauna of Lake Baikal-II. Unusual Lipid Composition of Two Sponge Species *Baicalospongia bacillifera* and *Baicalospongia intermedia* (Family Lubomirskiidae, Class Demospongiae), *Comp. Biochem. Physiol. 106B*, 825–831.
4. Dembitsky, V.M., Řezanka, T., and Kashin, A.G. (1994) Phospholipid and Fatty Acid Compositions of the Endemic Amphipod Crustacean *Issycogammarus bergi* from the Brackish Mountain Lake Issyk-Kul (Tian Shan, Middle Asia), *Comp. Biochem. Physiol. 107B*, 331–336.
5. Linko, R.R., and Karinkanta, H. (1970) Fatty Acids of Long Chain Length in Baltic Herring Lipids, *J. Am. Oil Chem. Soc. 47*, 42–46.
6. Řezanka, T. (1990) Analysis of Very Long Chain Polyenoic Fatty Acids by High Performance Liquid Chromatography and Gas Chromatography/Mass Spectrometry with Chemical Ionization, *LC–GC Intl. 3*, 46–49.
7. Ota, T., Kawabata, Y., and Ando, Y. (1994) Positional Distribution of 24:6(n-3) in Triacyl-*sn*-Glycerols from Flathead Flounder Liver and Flesh, *J. Am. Oil Chem. Soc. 71*, 475–478.
8. Ackman, R.G., and Hooper, S.N. (1974) Long Chain Monoethylenic and Other Fatty Acids in Heart, Liver, and Blubber

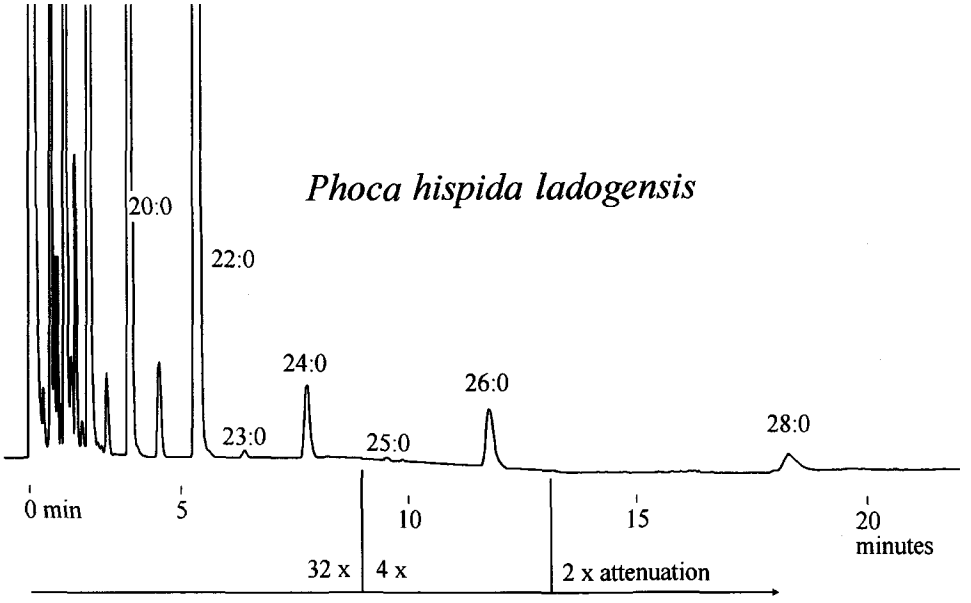


FIG. 3. Gas–liquid chromatogram of the hydrogenated total fatty acids from the blubber of the ringed seal of Lake Ladoga (isothermal 245°C, Omegawax-320 capillary column; Supelco, Bellefonte, PA).

Lipids of Two Harbor Seals (*Phoca Vitulina*) and One Grey Seal (*Halichoerus grypus*), *J. Fish. Res. Board Can. 31*, 333–341.
9. West, G.C., Burns, J.J., and Modafferi, M. (1979) Fatty Acid Composition of Blubber from the Four Species of Bering Sea Phocid Seals, *Can. J. Zool. 57*, 189–195.
10. Käkelä, R., Hyvärinen, H., and Vainiotalo, P. (1993) Fatty Acid Composition in Liver and Blubber of the Saimaa Ringed Seal (*Phoca hispida saimensis*) Compared with That of the Ringed Seal (*Phoca hispida botnica*) and Grey Seal (*Halichoerus grypus*) from the Baltic, *Comp. Biochem. Physiol. 105B*, 553–565.
11. Ackman, R.G. (1988) Some Possible Effects on Lipid Biochemistry of Differences in the Distribution on Glycerol of Long-Chain n-3 Fatty Acids in the Fats of Marine Fish and Marine Mammals, *Atherosclerosis 70*, 171–173.
12. Sprecher, H. (1992) A Reevaluation of the Pathway for the Biosynthesis of 4,7,10,13,16,19-Docosahexaenoic Acid, *Omega 3 News 7*, 1–3.
13. Aveldaño, M.I., Robinson, B.S., Johnson, D.W., and Poulos, A. (1993) Long and Very Long Chain Polyunsaturated Fatty Acids of the *n*-6 Series in Rat Seminiferous Tubules, *J. Biol. Chem. 268*, 11663–11669.
14. Ackman, R.G. (1967) Characteristics of the Fatty Acid Composition and Biochemistry of Some Fresh-Water Fish Oils and Lipids in Comparison with Marine Oils and Lipids, *Comp. Biochem. Physiol. 22*, 907–922.
15. Muje, P., Ågren, J.J., Lindqvist, O.V., and Hänninen, O. (1989) Fatty Acid Composition of Vendace (*Coregonus albula* L.) Muscle and Its Plankton Feed, *Comp. Biochem. Physiol. 92B*, 75–79.
16. Linko, R.R., Rajasilta, M., and Hiltunen, R. (1992) Comparison of Lipid and Fatty Acid Composition in Vendace (*Coregonus albula* L.) and Available Plankton Feed, *Comp. Biochem. Physiol. 103A*, 205–212.
17. Ozawa, A., Satake, M., and Fujita, T. (1993) Comparison of Muscle Lipid Composition between Marine and Landlocked Forms of Sockeye Salmon (*Oncorhynchus nerka*), *Comp. Biochem. Physiol. 106B*, 513–516.
18. Ahlgren, G., Blomqvist, P., Boberg, M., and Gustafsson, I.-B. (1994) Fatty Acid Content of the Dorsal Muscle—an Indicator of Fat Quality in Freshwater Fish, *J. Fish Biol. 45*, 131–157.
19. Ratnayake, W.M.N., Olsson, B., Matthews, D., and Ackman, R.G. (1988) Preparation of Omega-3 PUFA Concentrates from Fish Oils via Urea Complexation, *Fat Sci. Technol. 90*, 381–386.
20. Ackman, R.G. (1992) Application of Gas–Liquid Chromatography to Lipid Separation and Analysis: Qualitative and Quantitative Analysis, in *Fatty Acids in Foods and Their Health Implications* (Chow, C.K., ed.), pp. 47–63, Marcel Dekker, New York.
21. Ackman, R.G. (1972) The Analysis of Fatty Acids and Related Materials by Gas–Liquid Chromatography, in *Progress in the Chemistry of Fats and Other Lipids* (Holman, R.T., ed.) Vol. 12, pp. 165–284, Pergamon Press, Oxford.
22. Fellenberg, A.J., Johnson, D.W., Poulos, A., and Sharp, P. (1987) Simple Mass Spectrometric Differentiation of the n-3, n-6 and n-9 Series of Methylene Interrupted Polyenoic Acids, *Biomed. Environ. Mass Spectrom. 14*, 127–129.
23. Sokal, R.R., and Rohlf, F.J. (1981) *Biometry*, 2nd edn., pp. 427–428, W.H. Freeman and Company, San Francisco.
24. Ackman, R.G. (1994) α-Linolenic Acid in Human Adipose Tissue, *Lipids 29*, 445.
25. Shantha, N.C., and Ackman, R.G. (1991) Behaviour of a Common Phthalate Plasticizer (Dioctyl Phthalate) During the Alkali- and/or Acid-Catalyzed Steps in an AOCS Method for the Preparation of Methyl Esters, *J. Chromatogr. 587*, 263–267.
26. Ackman, R.G., Sebedio, J-L., and Kovacs, M.I.P. (1980) Role of Eicosenoic and Docosenoic Fatty Acids in Freshwater and Marine Lipids, *Mar. Chem. 9*, 157–164.
27. Řezanka, T. (1990) Identification of Very Long Polyenoic Acids as Picolinyl Esters by AG^+ Ion–Exchange High–Performance Liquid Chromatography, Reversed–Phase High Performance Liquid Chromatography and Gas Chromatography–Mass Spectrometry, *J. Chromatogr. 513*, 344–348.
28. Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H. (1991) The Metabolism of 7,10,13,16,19-Docosapentaenoic Acid to 4,7,10,13,16,19-Docosahexaenoic Acid in Rat Liver Is Independent of a 4-Desaturase, *J. Biol. Chem. 266*, 19995–20000.
29. Mayzaud, P., and Ackman, R.G. (1978) The 6,9,12,15,18-Heneicosapentaenoic Acid of Seal Oil, *Lipids 13*, 24–28.
30. Poulos, A. (1995) Very Long Chain Fatty Acids in Higher Animals–A Review, *Lipids 30*, 1–14.
31. Grogan, W.M. (1984) Metabolism of Arachidonate in Rat Testis: Characterization of 26–30 Carbon Polyenoic Acids, *Lipids 19*, 341–346.
32. Poulos, A., Sharp, P., Johnson, D., White, I., and Fellenberg, A. (1986) The Occurrence of Polyenoic Fatty Acids with Greater than 22 Carbon Atoms in Mammalian Spermatozoa, *Biochem. J. 240*, 891–895.
33. Poulos, A., Johnson, D.W., Beckman, K., White, I.G., and Easton, C. (1987) Occurrence of Unusual Molecular Species of Sphingomyelin Containing 28-34-Carbon Polyenoic Fatty Acids in Ram Spermatozoa, *Biochem. J. 248*, 961–964.
34. Poulos, A., Sharp, P., Johnson, D., and Easton, C. (1988) The Occurrence of Polyenoic Very Long Chain Fatty Acids with Greater Than 32 Carbon Atoms in Molecular Species of Phosphatidylcholine in Normal and Peroxisome-Deficient (Zellweger's Syndrome) Brain, *Biochem. J. 253*, 645–650.
35. Sharp, P., Johnson, D., and Poulos, A. (1991) Molecular Species of Phosphatidylcholine Containing Very Long Chain Fatty Acids in Human Brain: Enrichment in X-Linked Adrenoleukodystrophy Brain and Diseases of Peroxisome Biogenesis Brain, *J. Neurochem. 56*, 30–37.
36. Aveldaño, M.I. (1987) A Novel Group of Very Long Chain Polyenoic Fatty Acids in Dipolyunsaturated Phosphatidylcholines from Vertebrate Retina, *J. Biol. Chem. 262*, 1172–1179.
37. Aveldaño, M.I., and Sprecher, H. (1987) Very Long Chain (C_{24} to C_{36}) Polyenoic Fatty Acids of the n-3 and n-6 Series in Dipolyunsaturated Phosphatidylcholines from Bovine Retina, *J. Biol. Chem. 262*, 1180–1186.
38. Aveldaño, M.I. (1988) Phospholipid Species Containing Long and Very Long Polyenoic Fatty Acids Remain with Rhodopsin After Hexane Extraction of Photoreceptor Membranes, *Biochemistry 27*, 1229–1239.
39. Rotstein, N.P., and Aveldaño, M.I. (1988) Synthesis of Very Long Chain (up to 36 Carbon) Tetra, Penta and Hexaenoic Fatty Acids in Retina, *Biochem. J. 249*, 191–200.

[Received February 7, 1995, and in revised form June 5, 1995; Revision accepted June 6, 1995]

Cucumber Cotyledon Lipoxygenase Oxygenizes Trilinolein at the Lipid/Water Interface

K. Matsui* and T. Kajiwara

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan

ABSTRACT: The reactivity of cucumber cotyledon lipoxygenase with trilinolein was examined. The activity of the enzyme against linoleic acid rapidly decreased with increasing pH of the assay solution, and essentially no activity could be detected above pH 8.5. The rapid decrease in activity was not the result of an inactiveness of the enzyme at alkaline pH, because with trilinolein, the enzyme showed a broad pH-activity profile, and substantial activity could be detected even at pH 9.0. Rather, the decrease in activity was due to the dissociation of the linoleic acid emulsion into acid-soap aggregates and/or the monomeric form, depending on the ionization of the terminal carboxylic group. This suggests that cucumber cotyledon lipoxygenase acts only on an insoluble substrate at the lipid/water interface but not on a soluble one. High-performance liquid chromatography analyses of the products formed from trilinolein revealed that the enzyme inserted oxygen into the acyl moiety of trilinolein without hydrolysis of the ester bonds. Preincubation of the enzyme with triolein emulsions effectively abolished its activity against trilinolein added afterward. Furthermore, the enzyme was adsorbed on the trilinolein or triolein emulsion droplets in an essentially irreversible manner. A reaction velocity curve of the enzyme with trilinolein showed saturation kinetics. This is thought to be due to a regional substrate deficiency as the reaction proceeds. These lines of evidence indicate that the enzyme, once bound to the lipid/water interface, is unable to break free and bind to other emulsions.

Lipids 30, 733–738 (1995).

Lipoxygenases are enzymes containing a nonheme iron that use molecular oxygen in the oxygenation of a fatty acid having a 1Z,4Z-pentadiene system. The enzyme is ubiquitously distributed in animals and plants. In both animal and plant cells, lipoxygenases are involved in the formation of biologically active substances (1,2). It has been proposed that the formation of these compounds is controlled by the liberation of precursor fatty acids from membrane lipids *via* phospholipase- or lipolytic acyl hydrolase-catalyzed reactions (1,2). Thus, in general, lipoxygenases are thought to act on free fatty acids.

In the cucumber cotyledon, as well as in the cotyledons of other fatty seed plants, lipoxygenase is rapidly formed after germination, and then degrades after the cotyledons have acquired photosynthetic capacity (3,4). The developmental change in the lipoxygenase activity is highly correlated with that of seed storage fats, which are catabolized into sugars *via* gluconeogenesis (5). Thus, the involvement of lipoxygenase activity in the mobilization of fat stored in lipid bodies of seeds has been proposed (3,6). Nonetheless, free fatty acids are not so abundant during seed germination, and the actual reaction catalyzed by lipoxygenases *in vivo* has not yet been elucidated. It is well known that some soybean lipoxygenase isoforms having optimum pHs at neutral and weakly acidic regions act on triacylglycerols (6–8). If this is also the case with cucumber cotyledon lipoxygenase, it would give a new insight into the still-unknown physiological role of the enzyme in cotyledons. Thus, we examined whether a common triacylglycerol is a substrate for cucumber cotyledon lipoxygenase. In this report, the reactivity and kinetics of cucumber cotyledon lipoxygenase with trilinolein are described.

MATERIALS AND METHODS

Chemicals. Trilinolein, linoleic acid, and triolein (99% pure) were purchased from Sigma (St. Louis, MO). Gum arabic and Tween 20 were from Wako Pure Chemicals (Osaka, Japan). All other reagents were chemical grade. Stock solutions of trilinolein and triolein emulsions (25 mM) were prepared either with 50 mg/mL gum arabic (9) or 2 mg/mL Tween 20 after sonication at the highest power with a tip-type sonicator (model UR-150P; Tomy Co., Tokyo, Japan) for 30 s under a nitrogen atmosphere. The stock substrate solution was immediately frozen and kept at –20°C until use. For each assay, the substrate solution was frozen and thawed only once. Linoleic acid (50 mM) was dispersed with 2 mg/mL Tween 20 with sonication as described above and immediately frozen and kept at –20°C until use (4). In the case of linoleic acid/Tween 20, the stock solution was again sonicated just prior to use.

Enzyme and enzyme assays. Cucumber cotyledon lipoxygenase was purified from six-day-old light-grown cucumber cotyledons essentially as described (10). The purified lipoxygenase was apparently free of contaminants (see Fig. 5). The enzyme used in this study had a specific activity of more than

*To whom correspondence should be addressed.

Abbreviation: HPLC, high-performance liquid chromatography.

Copyright © 1995 by AOCS Press

2.0 μkat/mg protein, when assayed with trilinolein/gum arabic emulsions in 0.1 M Tris–Cl, pH 8.5. Although two forms of the lipoxygenases can be purified, only the 96 kDa form was used because it was thought to be an intact enzyme (10). Lipase activity was not detected in the lipoxygenase preparation when trilinolein or triolein was used as a substrate under the assay conditions described (9). Protein content was estimated according to Bradford (11) with bovine serum albumin as a standard.

The lipoxygenase assay was performed with an oxygen electrode (YSI 5331; Yellow Springs Instrument, Yellow Springs, OH) at 25°C (4). In a typical assay, 33.3 μL of 25 mM trilinolein/gum arabic solution was added to 0.1 M Tris–Cl, pH 8.5 (final volume was 1900 μL, and final concentrations of trilinolein and gum arabic were 0.439 mM and 0.877 mg/mL, respectively). With the linoleic acid/Tween 20 system, 50 μL of the stock solution was added, resulting in final concentrations of linoleic acid and Tween 20 of 1.32 mM and 52.6 μg/mL, respectively. After incubation of the solution for 1 min, the enzyme (114 ng; final concentration, 1.75 nM) was added to start the reaction. Although the reaction velocity curves with trilinolein and linoleic acid were considerably different (see Fig. 3A), the maximum activities that appeared within 5 min after addition of the enzyme were used to calculate the activity. Spectrophotometric analysis of the products was performed with a Shimadzu (Kyoto, Japan) UV–160A after extraction with ether. To estimate the effect of triolein on the activity, lipoxygenase (712 nM) was incubated with a given concentration of triolein/gum arabic emulsions in 0.05 M Tris–Cl, pH 8.5 at 25°C for 20 s, and a portion of the solution was used for the assay with trilinolein/gum arabic (final concentration of the enzyme was 1.5 nM).

High-performance liquid chromatography (HPLC) analysis. Trilinolein/gum arabic emulsions (0.438 mM and 0.877 mg/mL, respectively) were oxygenized with 1.5 nM lipoxygenase in 0.1 M Tris–Cl, pH 8.5 at 25°C for 8 min. The products were extracted from the reaction mixture (1.0 mL) with 2 mL of ether. After evaporation of the ether with a nitrogen flow, the residue was dissolved with 100 μL of methanol. A portion (10 μL) of the solution was separated with a Wakosil 5C18 column (Wako Pure Chemicals, 4 × 250 mm) attached to a Shimadzu HPLC pump LC-10A with 1-butanol/methanol (10:90, vol/vol) at a flow rate of 1.0 mL/min (12). Detection was performed with a photodiode array detector, Shimadzu SPD-M10A. HPLC analysis of the hydroperoxides of free fatty acids was performed with a Zorbax SIL (Dupont-Shimadzu, 4.6 mm × 250 mm) after reduction of the hydroperoxides with triphenylphosphine and methylation with ethereal diazomethane (13). Elution was carried out with *n*-hexane/*iso*-propanol (99:1, vol/vol) at a flow rate of 1 mL/min.

Differential centrifugation. The amount of the lipoxygenase bound to emulsion droplets was estimated after separation of the lipid phase with centrifugation. Cucumber cotyledon lipoxygenase (5.7 μg) was incubated with 12.5 mM of trilinolein or triolein emulsions dispersed with 25 mg/mL gum arabic in 0.1 M Tris–Cl, pH 8.5 (total volume, 200 μL) for 20 min on ice. It was then diluted to 3 mL with the same buffer, and was immediately centrifuged at 30,000 rpm for 20 min in a Hitachi (Tokyo, Japan) RPS-56T swing rotor at 4°C (14). The buffer layer was recovered from the bottom of the centrifugation tube. The remaining top lipid layer (300 μL) was thoroughly vortexed and collected. Recovery of the lipid from the respective phases was estimated from the turbidity which was determined from the absorption at 600 nm. A part of each solution (18.8 μL) was mixed with 14.3 μL of SDS–sample buffer, and then applied to SDS–polyacrylamide gel electrophoresis as in Reference 15. The protein bands were visualized by silver-staining with a Silver Staining Kit (Wako Pure Chemicals).

RESULTS

pH-activity curve. The reactivity of cucumber cotyledon lipoxygenase against triacylglycerol was estimated with trilinolein, which is the most abundant triacylglycerol species in dry cucumber seeds. The pH-activity profile of the lipoxygenase against trilinolein dispersed with gum arabic was compared with that against linoleic acid dispersed with Tween 20 (Fig. 1). When the linoleic acid/Tween 20 system was used as a substrate, high activities of cucumber cotyledon lipoxygenase were observed in the acidic pH region as reported previously (16). The activity rapidly decreased with increasing pH value of the buffer from 7.0 to 8.0. Essentially no activity was detected at or over pH 8.5. On the other hand, the lipoxygenase showed a relatively broad pH-activity profile with trilinolein/gum arabic. A substantial activity could be observed even at pH 9.0. This indicates that the low or absent activity of the lipoxygenase against linoleic acid/Tween 20 at alkaline pH was not due to the inactivity of the enzyme but to changes

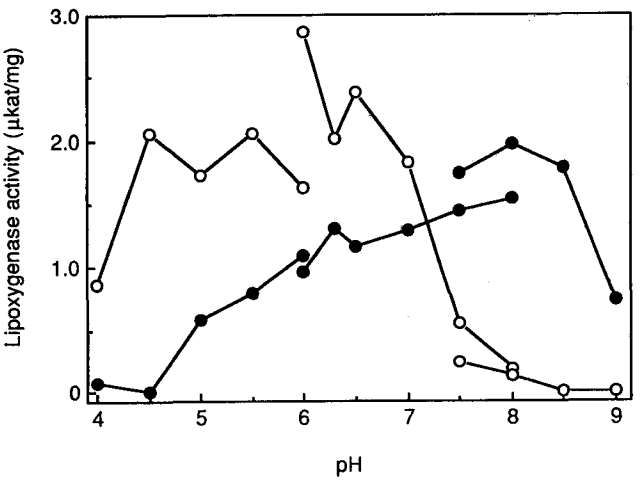


FIG. 1. The pH-activity profiles of cucumber cotyledon lipoxygenase with trilinolein emulsified with gum arabic (●) and with linoleic acid dispersed with Tween 20 (Wako Pure Chemicals, Osaka, Japan)(○). Buffers used were 0.1 M sodium acetate for pH 4.0 to 6.0, 0.1 M sodium phosphate for pH 6.0 to 8.0, and 0.1 M Tris–Cl for pH 7.5 to 9.0.

in the physical state of the substrate in the solution. Almost the same pH-activity profile was obtained when trilinolein was emulsified with Tween 20 (not shown). Bild *et al.* (17) reported the pKa of linoleic acid as about 7.9. Thus, an ionization of the terminal carboxyl group into its carboxylate anion form occurs above pH 7.0. Thus, an emulsion of linoleic acid should dissociate into acid–soap aggregates and/or into the monomeric form (18) as the buffer pH increases. In this respect, it is noteworthy that the pH-activity profile from pH 7.0 to 9.0 highly resembles the acid titration curve of sodium linoleate (17). On the contrary, the physical state of the emulsions of trilinolein should be little affected by the buffer pH because it has no ionizable group. These observations suggest that cucumber cotyledon lipoxygenase cannot act on a soluble substrate but only on an insoluble substrate at the water/lipid interface.

During the reaction of the lipoxygenase with trilinolein/gum arabic, the hydrolysis of the ester bond was not detected when the amounts of liberated fatty acid or its hydroperoxy derivatives were determined using the 1,5-diphenylcarbazide method (9). Spectrophotometric analysis revealed that during the reaction, a peak having a λ_{max} of 234 nm, which is typical of conjugated diene hydroperoxy groups, increased. Formation of the hydroperoxide of a free fatty acid could not be detected by analyzing the products formed from trilinolein with the straight-phase HPLC system. On the other hand, as shown in Figure 2, three peaks appeared in a chromatogram obtained with the reversed HPLC system, which was developed for separation of hydroperoxides of trilinolein (12). All three peaks showed spectra having a λ_{max} at 234 nm. Comparison of the elution profiles with published profiles (12), together with the spectra, allowed us to tentatively assign the peaks to tris-, bis-, and monohydroperoxide of trilinolein (in the elution order). These results suggest that oxygen is introduced into the acyl group of the triacylglycerol without hydrolysis.

Reaction velocity curves. Reaction velocity curves of the oxygenase reaction against linoleic acid/Tween 20 and trilinolein/gum arabic were completely different from each other (Fig. 3A). The enzyme oxygenized linoleic acid after a lag time with a progressively increasing reaction rate. The reaction velocity curve was similar at each pH tested and was not affected by the type of emulsifier used. This reaction velocity curve is typical of those reported for most lipoxygenases (19). The origin of this rate increase is explained with the two-step model for lipoxygenase catalysis (20). A lag time of 10–30 s was also observed with trilinolein, but, unlike the lag time ob-

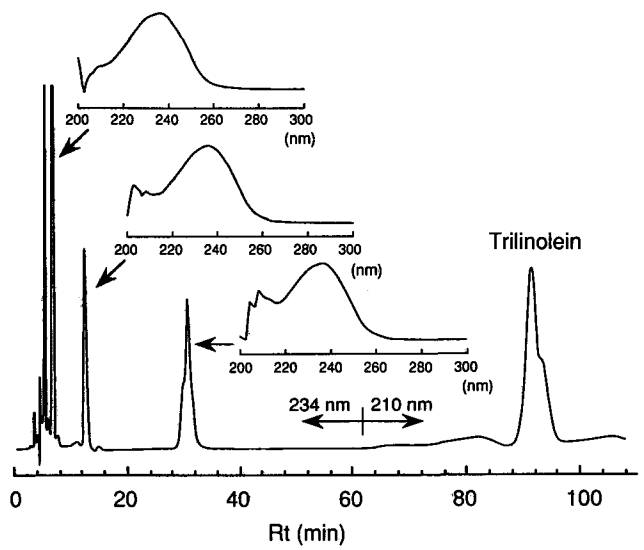


FIG. 2. A reversed-phase high-performance liquid chromatography analysis of the products formed from trilinolein with cucumber cotyledon lipoxygenase. Before 62 min, the eluate was monitored at 234 nm to detect the conjugated diene hydroperoxides. After that time, it was monitored at 210 nm to detect the ester bonds. Absorption spectra of newly appeared peaks (arrows) are also shown. Rt, retention time.

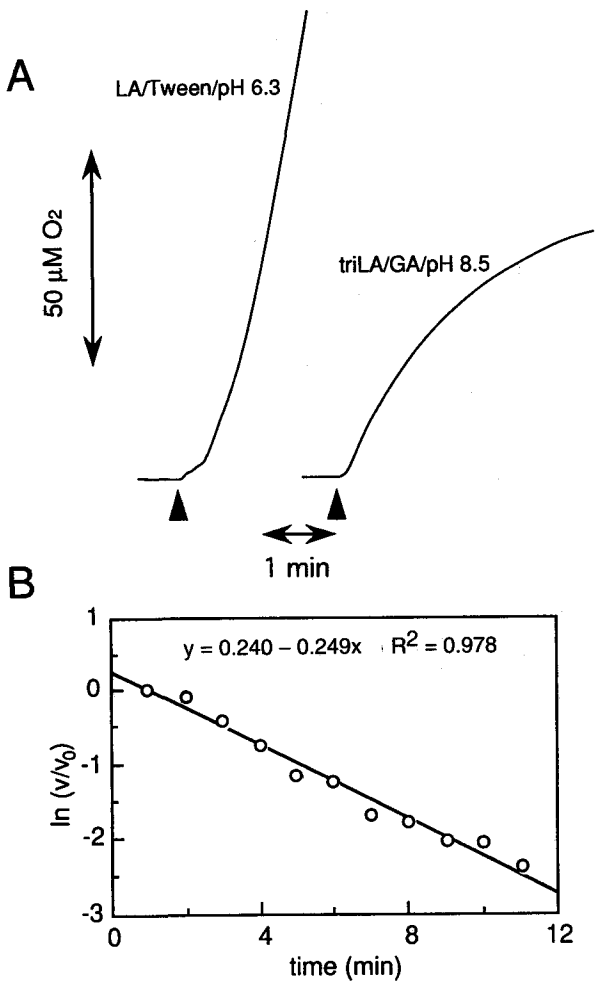


FIG. 3. Reaction velocity curves of cucumber cotyledon lipoxygenase. A: The curves of the lipoxygenase reactions were obtained polarographically with trilinolein (triLA)/gum arabic (GA) and linoleic acid(LA)/Tween 20. The times of enzyme addition are shown with arrowheads. B: Semi-log plot of the activity relative to the initial highest activity vs. time. The reaction rate with triLA/GA was traced every 60 s, and the natural logarithms of the rates at a given time relative to the initial highest rate are plotted. The regression curve does not intersect the zero point on the y-axis because the maximum activity was always observed after 10 to 30 s of the lag time. See Figure 1 for Company source.

served with linoleic acid, little reaction occurred during this period. After the lag time, the reaction rate rapidly increased and immediately reached its highest value. As the reaction proceeded, the rate slowly decreased, and finally the reaction almost ceased when only a fraction of the total available substrate was oxygenized. Such a reaction velocity curve is typical of Michaelis-Menten kinetics where the Michaelis constant is much larger than the concentration of a substrate, although the origin of the lag phase is not clear. Addition of fresh enzyme restarted the reaction with almost the same initial velocity. Thus, strong product inhibition should not be the reason for the cessation of the reaction. On the contrary, addition of trilinolein after the cessation of the reaction caused no change in the course of the reaction.

The decrease in the reaction rate at each point of time apparently obeyed the first-order kinetic law (Fig. 3B). The first-order exponential constant was independent of the initial concentrations of both the substrate and the enzyme as long as the trilinolein/enzyme ratio was kept between 300 and 2500. When a different concentration of the enzyme was used, the reaction began with the maximum velocity proportional to the amount of the enzyme added, and ceased after an amount of oxygen proportional to the initial amount of enzyme was consumed.

Adsorption on emulsions. Further evidence for determining whether cucumber cotyledon lipoxygenase catalyzes its reaction with trilinolein after the enzyme is adsorbed on emulsions could be obtained with triolein. Triolein is thought to form emulsions of almost the same physical properties as trilinolein but it is not a substrate of the lipoxygenase. When cucumber cotyledon lipoxygenase was preincubated with triolein/gum arabic emulsions, the activity was lost depending on the concentration of triolein (Fig. 4). The decrease in the activity observed here could be attributed to an essentially irreversible adsorption of the enzyme on the triolein emulsions. Because the adsorption process was completed within as little as 10 s, we could not follow the adsorption kinetics. From an extrapolation of the semi-log plot of the initial region of the inactivation curve, it can be calculated that one molecule of the lipoxygenase binds to approximately 4200 molecules of triolein (Fig. 4, inset).

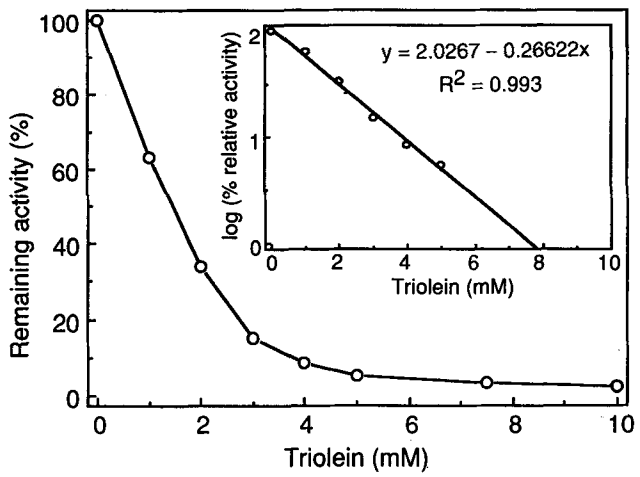


FIG. 4. Effect of preincubation of the enzyme with various concentrations of triolein/gum arabic emulsions on enzyme activity. The lipoxygenase was incubated with a given concentration of triolein/gum arabic, and a portion of the solution was used for the assay with trilinolein/gum arabic. The activity of the enzyme preincubated without triolein was 3.28 μkat/mg protein (designated as 100% in this figure). inset: Semi-log plot of the relative remaining activity. Only data obtained with a low concentration of triolein and which satisfactorily regressed to a straight line are shown.

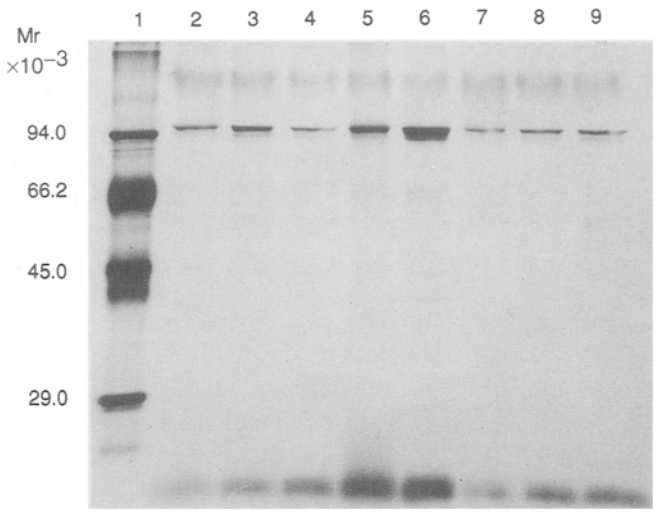


FIG. 5. Binding of cucumber cotyledon lipoxygenase to emulsion droplets. Lipoxygenase was incubated with an emulsion of trilinolein (lanes 2, 5, and 7) or triolein (lanes 3, 6, and 8). For a control, the lipoxygenase was incubated with the gum arabic solution without lipids (lanes 4 and 9). The preparations were then centrifuged, and the bottom buffer layer (lanes 7–9) and the top lipid layer (lanes 5 and 6) were separated. Lane 1, molecular mass standard; lanes 2–4, the solutions before centrifugation.

Binding of lipoxygenase to emulsion droplets. The emulsions were incubated with the enzyme, and then they were concentrated by floating with centrifugation. The turbidity assay indicated that trilinolein and triolein were concentrated 6.85- and 6.91-fold, respectively, in the top lipid phase, while they were diluted 4- and 3.2-fold, respectively, in the bottom buffer phase. The amounts of lipoxygenase recovered from the two phases were analyzed with SDS–polyacrylamide gel electrophoresis followed by silver-staining (Fig. 5). It is obvious that the enzyme was concentrated in the lipid phase and that the degrees of concentration were almost the same as those of the lipids. These results clearly indicate that almost all of the available lipoxygenase adsorbed onto the lipid emulsion droplets in an irreversible manner. Unexpectedly, the total recovery of the enzyme was much less with trilinolein emulsions than with triolein emulsions. The reason for such a low yield is presently unclear.

DISCUSSION

In both animal and plant cells, lipoxygenases are involved in the formation of various species of biologically active sub-

stances (1–3). It has been proposed that the formation of these compounds is controlled by the liberation of the precursor fatty acids from membrane lipids *via* phospholipase- or lipolytic acyl hydrolase-catalyzed reactions (21). Thus, lipoxygenases are thought to act essentially on free fatty acids. On the other hand, some lipoxygenase isoforms in soybean seeds are reported to act on an acyl moiety in glycerolipids (6–8). Furthermore, soybean lipoxygenase-1, which has a peculiar optimum pH, i.e., 9.0 to 10.0, has been reported to stereospecifically oxygenize an acyl moiety in phospholipids (22), although the catalysis required a specific bile salt–phospholipid complex instead of a monomer phospholipid (23). Reticulocyte lipoxygenase is also known to oxygenize biological membranes (24), and thus, involvement of lipoxygenase activity in the maturational breakdown of mitochondria during red cell maturation has been suggested (25). In this study, we demonstrated that cucumber cotyledon lipoxygenase oxygenizes trilinolein efficiently. Several lines of evidence indicated that the lipoxygenase acts only on a substrate at the lipid/water interface, and does not act on a substrate existing as a solute in an aqueous solution. The reaction velocity curve of the oxygenation on trilinolein was highly distinctive. Because the enzyme binds essentially irreversibly to the emulsions of trilinolein and, as a result, cannot jump from one emulsion droplet to another to sample the excess droplets, the cessation of the reaction can be attributed to the local depletion of substrates in the enzyme-containing emulsion droplets. Compared with trilinolein, the hydroperoxide derivatives of trilinolein are more hydrophilic and, thus, should remain on the surface of the emulsion. This is thought to be the reason why the local concentration of substrate that is accessible to cucumber cotyledon lipoxygenase became limited as the reaction proceeded. From these observations, it can be concluded that cucumber cotyledon lipoxygenase oxygenizes trilinolein at the lipid/water interface with the following sequence: the lipoxygenase (i) recognizes the interface, (ii) is irreversibly adsorbed on the surface, and (iii) oxygenizes one of the linoleates esterified to the glycerol moiety. Jain and Gelb (26) have developed the enzyme kinetics for the so-called scooting mode for several enzymes that act on lipids at the lipid/water interface. Although a precise definition of the properties of the substrate emulsions used in this study is needed, the catalysis by cucumber cotyledon lipoxygenase of trilinolein also may be assessed by scooting mode kinetics. Although there is no report on the scooting mode of action of any lipoxygenases, it should be noticed that most plant lipoxygenases investigated so far have optimum pH values at around 5.5–6.5 (2,3). At such pH values, linoleic and linolenic acids are thought to form aggregates under the assay conditions usually used. Thus, it is reasonable to expect that such plant lipoxygenases also act, at least preferentially, on substrates at the interface.

The observations described here raise two important questions about this enzyme. One, a biochemical question, is how the lipoxygenase differentiates a substrate at the lipid/water interface from one dissolved in aqueous solution. The enzyme cannot act on the monomeric form of linoleic acid but can act on the aggregated form. In order to facilitate such differentiation, the enzyme activity must be latent until it binds to the interface. Upon binding to the interface, the enzyme would be activated, probably by undergoing a structural change. Such a regulation of an enzyme activity after interfacial binding is known to occur with several kinds of enzymes, including human lipoprotein lipase (27), pig pancreatic phospholipase A_2 (28), human pancreatic lipase (29), and others. Although the precise tertiary structure of cucumber cotyledon lipoxygenase has not been reported, that of soybean lipoxygenase-1 has been reported (30). It is interesting that the tertiary structure of the N-terminal domain forms a β barrel similar to that of the COOH-terminal portion of human pancreatic lipase (29). Such an adsorption-mediated activation mechanism, if present in cucumber cotyledon lipoxygenase, might be more complicated than these other examples, because in the case of lipoxygenase, product activation that accompanies oxidation of the enzyme from the Fe(II) form to the Fe(III) form (20) should be taken into consideration.

The second question is whether the enzyme's activity on triacylglycerol has any physiological significance. Developmental changes in cucumber cotyledon lipoxygenase activity after germination of the seeds showed a high correlation with the degradation rate of seed storage fats (3,4). Thus, it is expected that the enzyme's activity on triacylglycerol has significance in the mobilization of the carbon and in the production of energy from stored fats. In fact, a lipid body in cucumber cells is reported to bind lipoxygenase on its surface (31). Nonetheless, it has been widely accepted that the stored fats are degraded sequentially by lipase hydrolysis, β-oxidation, and gluconeogenesis (5). Recently, it has been reported that a lipid hydroperoxide is a better substrate than unoxidized lipid for a plant phospholipase (32). Thus, it is expected that oxygenation of triacylglycerols in lipid bodies by cucumber cotyledon lipoxygenase might be a trigger for hydrolysis. In order to test this hypothesis, properties of cucumber cotyledon lipase have to be examined in detail.

ACKNOWLEDGMENT

This work was partly supported by the Grant-in Aid for Scientific Research on Priority Areas No. 06240102 from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Yamamoto, S. (1992) Mammalian Lipoxygenases: Molecular Structures and Functions, *Biochim. Biophys. Acta 1128*, 117–131.
2. Siedow, J.N. (1991) Plant Lipoxygenase: Structure and Function, *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42*, 145–188.
3. Vick, B.A., and Zimmerman, D.C. (1987) Oxidative Systems for Modification of Fatty Acids: The Lipoxygenase Pathway, in *The Biochemistry of Plants* (Stumpf, P.K., and Conn, E.E., eds.) Vol. 9, pp. 53–90, Academic Press, London.
4. Matsui, K., Irie, M., Kajiwara, T., and Hatanaka, A. (1992) Developmental Changes in Lipoxygenase Activity in Cotyledons of Cucumber Seedlings, *Plant Sci. 85*, 23–32.

5. Beevers, H. (1979) Microbodies in Higher Plants, *Ann. Rev. Plant Physiol. 30*, 159–193.
6. Holman, R.T. (1948) Lipoxidase Activity and Fat Composition of Germinating Soy Beans, *Arch. Biochem. Biophys. 17*, 459–466.
7. Koch, R.B., Stern, B., and Ferrari, C.G. (1958) Linoleic Acid and Trilinolein as Substrates for Soybean Lipoxidase(s), *Arch. Biochem. Biophys. 78*, 165–179.
8. Zhuang, H., Hildebrand, D.F., Andersen, R.A., and Hamilton-Kemp, T.R. (1991) Effects of Polyunsaturated Free Fatty Acids and Esterified Linoleoyl Derivatives on Oxygene Consumption and C6-Aldehyde Formation with Soybean Seed Homogenates, *J. Agric. Food Chem. 39*, 1357–1364.
9. Wang, S., and Huang, A.H.C. (1987) Biosynthesis of Lipase in the Scutellum of Maize Kernel, *J. Biol. Chem. 262*, 2270–2274.
10. Matsui, K., Irie, M., Kajiwara, T., Kakuno, T., and Hatanaka, A. (1993) Rapid Degradation of Cucumber Cotyledons Lipoxygenase, *Phytochemistry 32*, 1387–1391.
11. Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*, 248–254.
12. Akasaka, K., Ijichi, S., Watanabe, K., Ohrui, H., and Meguro, H. (1992) High-Performance Liquid Chromatography and Post-Column Derivatization with Diphenyl-1-Pyrenylphosphine for Fluorimetric Determination of Triacylglycerol Hydroperoxides, *J. Chromatgr. 596*, 197–202.
13. Matsui, K., Shinta, H., Toyota, H., Kajiwara, T., and Hatanaka, A. (1992) Comparison of the Substrate Specificities of Lipoxygenases Purified from Soybean Seed, Wheat Seed, and Cucumber Cotyledons, *Z. Naturforsch. 47c*, 85–89.
14. Bengtsson, G., and Olivecrona, T. (1981) Lipoprotein Lipase: Modification of Its Kinetic Properties by Mild Tryptic Digestion, *Eur. J. Biochem. 113*, 547–554.
15. Laemmli, U.K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, *Nature 227*, 680–685.
16. Matsui, K., Kajiwara, T., Hayashi, K., and Hatanaka, A. (1988) Tissue Specific Heterogeneity of Lipoxygenase in Cucumber Seedlings, *Agric. Biol. Chem. 52*, 3219–3221.
17. Bild, G.S., Ramadoss, C.S., and Axelrod, B. (1977) Effect of Substrate Polarity on the Activity of Soybean Lipoxygenase Isoenzymes, *Lipids 12*, 732–735.
18. AlSheikhly, M., and Simic, M.G. (1989) Chain-Propagation Length of Linoleic Acid Peroxidation in Aqueous Monomeric and Micellar Systems, *J. Phys. Chem. 93*, 3103–3106.
19. Clapp, C.H., Banerjee, A., and Rotenberg, S.A. (1985) Inhibition of Soybean Lipoxygenase 1 by *N*-Alkylhydroxylamines, *Biochemistry 24*, 1826–1830.
20. Schilstra, M.J., Veldink, G.A., and Vliegenthart, J.F.G. (1994) The Dioxygenation Rate in Lipoxygenase Catalysis is Determined by the Amount of Iron (III) Lipoxygenase in Solution, *Biochemistry 33*, 3974–3979.
21. Farmer, E.E., and Ryan, C.A. (1992) Octadecanoid Precursors of Jasmonic Acid Activate the Synthesis of Wound-Inducible Proteinase Inhibitors, *Plant Cell 4*, 129–134.
22. Brash, A.R., Ingram, C.D., and Harris, T.M. (1987) Analysis of a Specific Oxygenation Reaction of Soybean Lipoxygenase-1 with Fatty Acids Esterified in Phospholipids, *Biochemistry 26*, 5465–5471.
23. Eskola, J., and Laakso, S. (1983) Bile Salt-Dependent Oxygenation of Polyunsaturated Phosphoatidylcholines by Soybean Lipoxygenase-1, *Biochim. Biophys. Acta 751*, 305–311.
24. Kuhn, H., Belkner, J., Wiesner, R., and Brash, A.R. (1990) Oxygenation of Biological Membranes by the Pure Reticulocyte Lipoxygenase, *J. Biol. Chem. 265*, 18351–18361.
25. Rapoport, S.M., and Schewe, T. (1986) The Maturational Breakdown of Mitochondria in Reticulocytes, *Biochim. Biophys. Acta 864*, 471–495.
26. Jain, M.K., and Gelb, M.H. (1991) Phospholipase A2-Catalyzed Hydrolysis of Vesicles: Uses of Interfacial Catalysis in the Scooting Mode, *Method. Enzymol. 197*, 112–125.
27. Wong, H., Davis, R.C., Thuren, T., Goers, J.W., Nikazy, J., Waite, M., and Schotz, M.C. (1994) Lipoprotein Lipase Domain Function, *J. Biol. Chem. 269*, 10319–10323.
28. Maliwal, B.P., Yu, B.-Z., Szmacinski, H., Squier, T., Binsbergen, J., Slotboom, A.J., and Jain, M.K. (1994) Functional Significance of the Conformational Dynamics of the N-terminal Segment of Secreted Phospholipase A2 at the Interface, *Biochemistry 33*, 4509–4516.
29. Winkler, F.K., D'Arcy, A., and Hunziker, W. (1990) Structure of Human Pancreatic Lipase, *Nature 343*, 771–774.
30. Boyington, J.C., Gaffney, B.J., and Amzel, L.M. (1993) The Three-Dimensional Structure of an Arachidonic Acid 15-Lipoxygenase, *Science 260*, 1482–1486.
31. Radetzky, R., Feussner, I., Theimer, R.R., and Kindl, H. (1993) Transient Occurrence of Lipoxygenase and Glycoprotein gp49 in Lipid Bodies During Fat Mobilization in Anise Seedlings, *Planta 191*, 166–172.
32. Banas, A., Johansson, I., and Stymne, S. (1992) Plant Microsomal Phospholipases Exhibit Preference for Phosphatidylcholine with Oxygenated Acyl Groups, *Plant Sci. 84*, 137–144.

[Received February 27, 1995, and in revised form May 2, 1995; Revision accepted May 15, 1995]

Critical Analysis of Phospholipid Hydrolyzing Activities in Ripening Tomato Fruits. Study by Spectrofluorimetry and High-Performance Liquid Chromatography

Marie-Aude Rouet-Mayer[a], Olga Valentova[b], Elizabeth Simond-Côte[a], Jean Daussant[a] and Claudine Thévenot[a,]*

[a]Laboratoire de Physiologie des Organes Végétaux après Récolte et Université Pierre et Marie Curie, 92190 Meudon, France and [b]Institute of Chemical Technology, 166 28 Prague, Czech Republic

ABSTRACT: Using the spectrofluorimetric method described by Wittenauer *et al.* [Wittenauer, L.A., Shirai, K., Jackson, R.L., and Johnson, J.D. (1984) *Biochem. Biophys. Res. Commun. 118,* 894–901] for phospholipase A_2 (PLA_2) measurement, we have detected a phospholipase activity in Ailsa Craig and in mutant *rin* tomatoes at their normal harvest time (mature green stage). This activity in Ailsa Craig tomatoes increased at the beginning of fruit ripening (green–orange stage) and then decreased slowly. The decrease in activity, however, was greater when ripening occurred after tomato picking at normal harvest time than when ripening occurred on tomato plants. This phospholipase activity was always higher in *rin* tomatoes than in normal ones. Thin-layer chromatography of compounds obtained after incubation of tomato extract demonstrated a decrease in the substrate 1-acyl-2-{6[(7-nitro-2,1,3, benzoxadiazol-4-yl)amino]-caproyl}-*sn*-glycero-3-phosphocholine (C_6-NBD-PC), and an increase in one product (NBD-aminohexanoic acid), but failed to detect the second product (1-acyl-*sn*-glycero-3-phosphocholine). We, therefore, developed a new one-step method for separation and quantification of a mixture of phospholipids and other lipids, using straight-phase–high-performance liquid chromatography with light-scattering detection. This method detected another fatty acid-releasing activity in enzyme extract from green–orange tomatoes. This lipolytic enzyme (or family of enzymes) slowly produced free fatty acids when 1-oleoyl-*sn*-glycero-3-phosphocholine was added as substrate. The production of fatty acids was stoichiometric and more rapid when 1-oleoyl-*sn*-glycero-3-phosphate and 1-oleoyl-*sn*-glycerol were used as substrates. On the other hand, the same tomato extract was unable to hydrolyze 1,2-dioleoyl-*sn*-glycero-3-phosphate and 1,2-dioleoyl-*sn*-glycerol. Crude tomato extract exhibited lipid acyl hydrolase activity according to the definition of Galliard [Galliard, T. (1979) in *Advances in the Biochemistry and Physiology of Plant Lipids* (Appelqvist, L.A., and Liljenberg, C. eds.) pp. 121–132, Elsevier, Amsterdam]. But in order to demonstrate whether tomato extract contains PLA_2 activity and/or lysophospholipase activity, further work on purified tomato extract will be necessary.
Lipids 30, 739–746 (1995).

Decrease in phospholipid content usually characterizes the onset of senescence in a wide range of plant tissues (1). In tomato fruits, Güçlü *et al.* (2) have shown that the level of total phospholipids increases during ripening and then decreases drastically.

Phospholipases are enzymes which generally catalyze the hydrolysis of phospholipids, and are classified according to the bond cleaved. Thus phospholipase A_1 (PLA_1; EC 3.1.1.3), which was not described until now in higher plants, and phospholipase A_2 (PLA_2; EC 3.1.1.4), catalyze the hydrolysis of the *sn*-1 or *sn*-2 position fatty acyl ester bond of phosphoglycerides. Lysophospholipase (EC 3.1.1.5) cleaves the single fatty acid ester bond of a lysophospholipid. In contrast to animal and microbial phospholipases, which seem to be quite specific for phospholipid substrate, no such property has been clearly demonstrated in plants (3–5), where a lipid acyl hydrolase has often been described. This enzyme most rapidly catalyzes hydrolysis at the *sn*-1 and *sn*-2 positions of the fatty acyl ester bond of phospholipids, lysophospholipids, and monoacylglycerols (4). Two other phospholipases have been described: phospholipase C (PLC; EC 3.1.4.3), which cleaves the *sn*-3 phosphodiester bond of a diacylglycerophospholipid on the glycerol side, and phospholipase D (PLD; EC 3.1.4.4), which catalyzes hydrolysis of the same bond between the phosphatidic acid and the base represented by choline, ethanolamine, glycerol, or inositol. The products obtained after hydrolysis with these enzymes are free fatty acids, lysophospholipids, diacylglycerol, and/or phosphatidic acid.

Among the enzymes responsible for lipid deterioration, lipoxygenases (LOX) peroxidize unsaturated fatty acids. Droillard *et al.* (6) have shown that LOX levels increase during tomato ripening and then decrease after the green–orange stage of maturation. They also demonstrated that free fatty

*To whom correspondence should be addressed at Institut de Biotechnologie des Plantes, Université de Paris Sud, Bâtiment 630, 91405 Orsay Cedex, France.

Abbreviations: C6-NBD-PC, 1-acyl-2-{6[(7-nitro-2,1,3,benzoxadiazol-4-yl)amino]-caproyl}-*sn*-glycero-3-phosphocholine; LOX, lipoxygenase; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; SP-HPLC, straight-phase high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet.

Copyright © 1995 by AOCS Press

acids were better substrates for LOX than fatty acids esterified in phospholipids. In ripening tomato fruits, we studied PLA_2, which is able to release free fatty acids, the substrate for LOX activity. In ripening tomato fruits, phosphatidylcholine is the most important class among phospholipids and contains five different fatty acids: palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid, whose percentages vary during ripening (2). For our study of phospholipid hydrolysis in tomato fruits, we chose 1,2-dioleoyl-*sn*-glycero-3-phosphocholine as substrate in order to avoid LOX oxidation of the released free fatty acids.

PLA_2 has been widely studied in the animal kingdom, especially secreted PLA_2 from venoms and pancreas, because of their high activity. But the activities of intracellular and membrane-bound PLA_2 are very low. Two kinds of sensitive assays are therefore currently used. The most common assay involves the use of a phospholipid with a ^{14}C-labeled fatty acid in the *sn*-2 position, but this method is time-consuming and requires the use of expensive radiolabeled lipids. Because of the high sensitivity of fluorescence, Wittenauer *et al.* (7) used a fluorescent phospholipid, 1-acyl-2-{6[(7-nitro-1,2,3,benzoxadiazol-4-yl)amino]-caproyl}-*sn*-glycero-3-phosphocholine (C_6-NBD-PC), as substrate. This method is rapid and does not require any lipid extraction. It appears to be especially useful for applications, such as monitoring PLA_2 activity during maturation of tomato fruits.

As pointed out by Wittenauer *et al.* (7), Hendrickson *et al.* (8), and Moreau (9), this method is not really specific because of other phospholipases which hydrolyze C_6-NBD-PC with fluorescence enhancement. When the enzymatic extract is likely to contain different phospholipase activities or unknown activities, it is therefore also necessary to analyze the products after hydrolysis of the substrate. Consequently, we have devised a method which uses straight-phase high-performance liquid chromatography (SP–HPLC) with evaporative light-scattering detection to identify and quantify substrate and products after hydrolysis of phospholipids by tomato extract.

MATERIALS AND METHODS

Plant material. Tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig and mutant *rin*) plants were grown in a heated greenhouse under natural light. The various stages of ripeness [mature green stage (G), green–orange (GO), orange (O), and red (R)] were determined by measurement of tomato fruit coloration with a Minolta C 100 chromameter (Minolta France S.A., Colombes, France). Mutant green ("G") fruits were harvested when the normal fruits had reached the mature green (G) stage. Since mutant *rin* tomatoes were unable to ripen, the last stage "R" was chosen at the time when the normal fruits became red.

In order to compare natural ripening on tomato plants and post-harvest ripening, mature green tomatoes (normal and mutant) were harvested and kept at 20°C. The various stages of ripeness (GO, O, and R) were determined by tomato coloration.

Reagents. C_6-NBD-PC was obtained from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-(1^{14}C)-palmitoyl-*sn*-glycero-3-phosphocholine was obtained from Amersham France (Les Ulis, France). Porcine pancreatic PLA_2 (600 units/mg protein), NBD-aminohexanoic acid and usual lipids were obtained from Sigma (St. Louis, MO). All solvents (HPLC grade) were obtained from Prolabo (Paris, France).

Preparation of tomato extracts. Three tomatoes were peeled, and fresh pericarp tissue was extracted in cold acetone (–20°C). The lipid-free powder was suspended in 100 mM HEPES buffer (pH 7.5) and leupeptin. After homogenization, filtration, and centrifugation, the supernatant was brought to 50% saturation in ammonium sulfate. The pellet was resuspensed in 100 mM HEPES buffer (pH 7.5) and desalted on a PD-10 column (Pharmacia, Uppsala, Sweden). Of the initial activity, 70% was recovered. All extraction procedures were carried out at 4°C. Ammonium sulfate precipitate could be stored at –20°C for more than five days with a very small decrease in activity.

PLA_2 assays. 1. Spectrofluorimetric assays. These were carried out essentially as described by Wittenauer *et al.* (7). The ethanolic solution of C_6-NBD-PC was injected into the thermostated fluorimetric cuvette in 50 mM HEPES buffer pH 7.1 or in 30 mM HEPES–Tris buffer pH 8.5 (5 μM of C_6-NBD-PC in a final volume of 2 mL). The emission spectrum was checked on this mixture. After a 3-min preincubation, the assay was initiated by adding 10–50 μL of tomato extract. The relative fluorescence was monitored continuously (for 10 min) with constant stirring using an Alphascan System from PTI (excitation 466 nm, emission 552 nm; Photon Technology International, Inc., South Brunswick, NJ). No relative fluorescence was found with a heat-deactivated tomato extract.

2. Radiochemical assays. These were carried out essentially as described by Palmgren *et al.* (10) and Vignon *et al.* (11), with some modifications. 1,2-diacyl-*sn*-glycero-3-phosphocholine, type X-E, from egg yolk, was dissolved in ethanol and homogenized with labeled 1-palmitoyl-2-(1^{14}C)-palmitoyl-*sn*-glycero-3-phosphocholine. Aliquots containing about 0.02 μCi were transferred into test tubes and the solvent removed. Sodium deoxycholate, $CaCl_2$, and 30 mM HEPES–Tris pH 8.5 were added before tomato extract addition and incubated at 37°C. After 10 min, the reaction was stopped, the released free fatty acids extracted, and their radioactivity was counted. PLA_2 activity was calculated from the amount of labeled fatty acid produced, with one unit defined as 1 μmol of ^{14}C-palmitoyl acid released per minute.

Analysis of hydrolytic products by thin-layer chromatography (TLC). This analysis was carried out essentially as described by Wittenauer *et al.* (7). The reaction mixture containing 5 μM of C_6-NBD-PC, 30 mM HEPES–Tris buffer, pH 8.5, 10 mM of $CaCl_2$ was injected with dialyzed PLA_2 from porcine pancreas (3.66 μg/mL of reaction mixture) or with tomato fruit extract (400 μL/mL of reaction mixture). The incubation was performed at 37°C, with magnetic stirring, for 1 h. Aliquots were taken out after 30 and 60 min and vigorously shaken in a methanol/chloroform/heptane

mixture (1.42:1.25:1, by vol). Each phase—phase a (aqueous) and phase o (organic)—was spotted on silica gel plates. The plates were developed in a solvent system of chloroform/methanol/water (65:25:4, by vol). C_6-NBD-PC and NBD-aminohexanoic acid were detected with an ultraviolet (UV) lamp. The other phospholipid and lipid spots were examined under UV light after being sprayed with Primulin (Sigma).

Characterization and quantification of 1,2-dioleoyl-sn-glycero-3-phosphocholine and hydrolytic products by HPLC. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (50 µL of chloroform solution—20 mg per mL) was placed in a test tube, and the solvent was evaporated under nitrogen. One mL of 30 mM HEPES–Tris buffer (pH 8.5) containing 0.3% deoxycholate was added to the dried lipid and vortexed vigorously.

The reaction mixture (final volume: 1 mL) containing 400 µL of tomato extract, 10 mM $CaCl_2$, 150 µM heptadecanoic acid, i.e., internal standard, and 100 µL of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine emulsion in 30 mM HEPES–Tris buffer (pH 8.5) was incubated at 37°C with magnetic stirring.

At various time intervals, 0.2 mL of assay mixture was removed, and lipids were extracted from the different samples according to the procedure of Bligh and Dyer (12). The chloroform layer (0.5 mL) was dried under a nitrogen stream, and the dried sample was resolubilized in 0.2 mL of chloroform; 20 µL of the solution was analyzed by SP–HPLC.

SP–HPLC was carried out with an Ultrasphere silica (5 µm) column (250 x 4.6 mm, Beckman, Fullerton, CA). Two mobile phases were prepared: phase A, chloroform/methanol/ammonia (84:15.5:0.5, by vol) and phase B, chloroform/methanol/water/ammonia (60:34:5.5:0.5, by vol). The column was equilibrated with 100% phase A. The flow rate of the eluent was kept constant at 1 mL/min at room temperature. The solvent program was: 13 min 0% phase B, 6 min 0–100% phase B, 12 min 100% phase B. The effluent was monitored using an evaporative light-scattering detector/DDL 21 (Cunow, Cergy Pontoise, France) with a nebulization temperature of 50°C and a carrier gas (air) pressure of 1.8 bar.

Gas chromatography. Lipids were extracted with Dole's reagent (propanol/heptane/1 N H_2SO_4; 4:1:0.1, by vol) before methylation according to Metcalfe *et al.* (13). For quantitative determination, a known amount of heptadecanoic acid was added as an internal standard. The methylated fatty acid esters were analyzed on a Girdel (Suresnes, France) type 30 gas chromatograph, using a Carbowax column 0.32 mm in diameter and 25 m long. The carrier gas was helium, the column temperature 165°C, the detector temperature 250°C, and the injection temperature 230°C.

RESULTS

Measurement of phospholipase activity in ripening tomato fruits by spectrofluorimetric assay. The activity increased between stages G and GO and then decreased when the fruits were submitted to ripening on tomato plants or post-harvest ripening (Fig. 1). Activities measured in post-harvest tomatoes were always slightly lower than those in fruits ripened on tomato plants, but the variability from one harvest to another was higher when the fruits were post-harvest ripened.

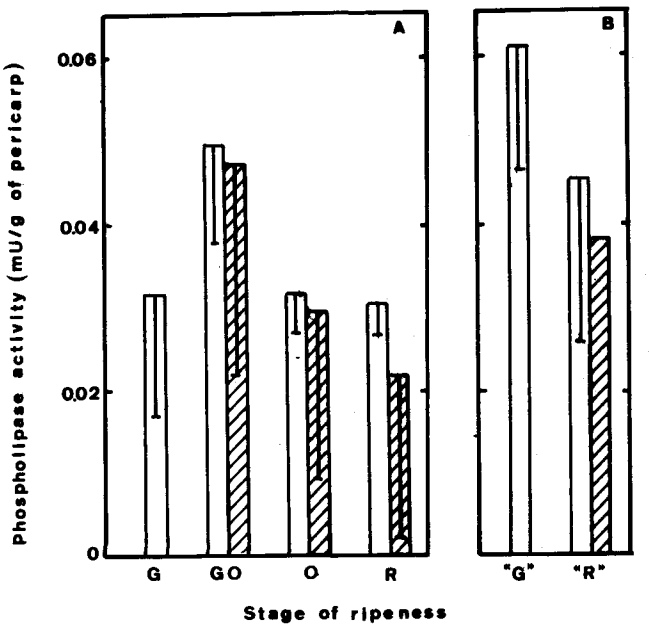


FIG. 1. Phospholipase activity, measured by spectrofluorimetry in normal (A) and in mutant fruits (B), allowed to ripen on tomato plants (white bar) or after harvest at green stages and keeping at 20°C (striped bar). The values are the average with standard deviation of three tomato harvests; G, green; GO, green–orange; O, orange; R, red; "G," mutant green; "R," mutant fruits chosen at the time when the normal fruit becomes red.

The activity in mutant green ("G") fruits was very high in comparison with the normal green (G) tomatoes. In the mutant *rin* fruits, phospholipase activity decreased more when the fruits were post-harvest ripened at 20°C than when they were kept on tomato plants. Surprisingly, activity was higher in mutant *rin* tomatoes, which were unable to ripen, than in ripening normal fruits.

In order to characterize this phospholipase activity, all further assays use extracts from normal tomatoes harvested at the GO stage.

Optimization of spectrofluorimetric assays. The activity of a tomato fruit extract in a fluorimetric cuvette measured by relative fluorescence was multiplied by about 2.5 when the temperature was increased from 25 to 37°C and the pH from 7.1 to 8.5 (Table 1). The activity of a solution of porcine pancreatic PLA_2 increased noticeably at 37°C and pH 8.5 when 10 mM $CaCl_2$ was added. The addition of 0.03% sodium deoxycholate enhanced this increase (results not shown). With tomato extract, this increase was not observed either with $CaCl_2$ or with sodium deoxycholate and $CaCl_2$. In order to determine the true nature of the phospholipase activity revealed by the spectrofluorimetric method, we analyzed the products of the incubation reaction of C_6-NBD-PC with tomato extract.

Study of the products obtained during spectrofluorimetric assays. The products obtained after incubation of C_6-NBD-

TABLE 1
Effect of Temperature and pH on Phospholipase Activity[a]

	Temperature		
	25°C	30°C	37°C
pH 7.1	159	198	207
pH 8.5	260	284	384

[a]From tomato fruits determined by spectrofluorimetric assay. The results are expressed by variation of relative fluorescence for 1000 s. Experimental conditions as described in the Materials and Methods section.

PC with PLA_2 or tomato extract were analyzed by TLC. When C_6-NBD-PC was incubated with porcine pancreatic PLA_2, the intensity of the spot which corresponds to C_6-NBD-PC in the organic phase (Fig. 2B) decreased with time. In the aqueous phase, two spots appeared, one near the solvent front corresponding to NBD-aminohexanoic acid (compare with Fig. 2A, line 6), and the second near the starting line corresponding to 1-oleoyl-*sn*-glycero-3-phosphocholine (compare with Fig. 2A, line 2).

When C_6-NBD-PC was incubated with tomato extract, the decrease in C_6-NBD-PC was observed as the increase in NBD-aminohexanoic acid (Fig. 2C). However, instead of a spot of 1-oleoyl-*sn*-glycero-3-phosphocholine, two unidentified spots appeared in the aqueous phase and one in the organic phase. This result could square with the presence in the tomato extract of a family of enzyme able to hydrolyze different moieties of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. We therefore developed an SP–HPLC method for one-step separation of a mixture of phospholipids and other lipids.

*SP–HPLC determination of the products of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine hydrolysis by porcine pancreatic PLA_2.* Incubation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine with porcine pancreatic PLA_2 gave, within 35 min and after extraction of lipids, a typical profile as shown in Figure 3. The substrate, i.e., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, and both products, i.e., oleic acid and 1-oleoyl-*sn*-glycero-3-phosphocholine, were separated in one step.

In order to confirm the identity and quantify the peaks, standard solutions of heptadecanoic acid, oleic acid, 1-oleoyl-*sn*-glycero-3-phosphocholine, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine were injected into the chromatographic system.

The concentrations of the different compounds found in the reaction mixture containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and porcine pancreatic PLA_2 were determined by SP–HPLC using the calibration curves (Fig. 4). An excellent stoichiometry between the digested 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and both products of hydrolysis by PLA_2 was obtained for high concentrations (>100 μM) of oleic acid and 1-oleoyl-*sn*-glycero-3-phosphocholine produced (Fig. 5). For lower concentrations, oleic acid and 1-oleoyl-*sn*-glycero-3-phosphocholine are probably undervalued because of the lower sensitivity of our method for these products. The validity of values obtained by SP–HPLC for oleic acid was checked using gas chromatography. In the reaction mixture containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and PLA_2, two aliquots were taken, one for SP–HPLC determination, which was extracted according to the procedure of Bligh and Dyer (12), and one for gas–liquid chromatography, which was extracted with Dole's reagent before methylation. The values obtained were the same (results not shown). For SP–HPLC, the detection limit of oleic acid was 170 ng, i.e., 30 μM. The coefficient of variation, for 8 injections of 150 μM of heptanoic acid, i.e., internal standard, was about 8%. Gas chromatography was found to be about 10-fold more sensitive than SP–HPLC and the coefficient of variation was about 3%.

*SP–HPLC determination of the products of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine hydrolysis by tomato extract.*

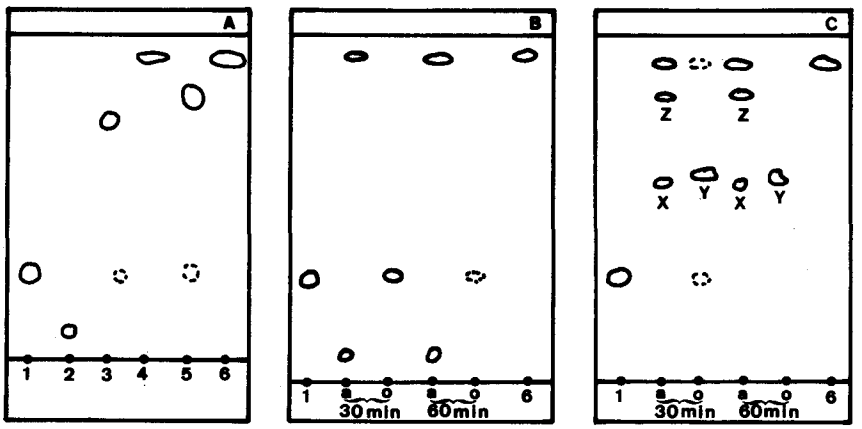


FIG. 2. Thin–layer chromatogram on silica gel plates. Standards in MeOH (A). Separation of 1-acyl-2-{6[(7-nitro-2,1,3,benzoxadiazol-4-yl)amino]-caproyl}-*sn*-glycero-3-phosphocholine (C_6-NBD-PC) hydrolysis products after 30 and 60 min of incubation with porcine pancreatic phospholipase A_2 (B) and tomato extract (C), a = aqueous phase; o = organic phase. 1 = C_6-NBD-PC; 2 = 1-oleoyl-*sn*-glycero-3-phosphocholine; 3 = 1,2-dioleoyl-*sn*-glycero-3-phosphate; 4 = 1-oleoyl-*sn*-glycerol; 5 = 1-oleoyl-*sn*-glycero-3-phosphate; 6 = NBD-aminohexanoic acid; x,y,z = unknown products.

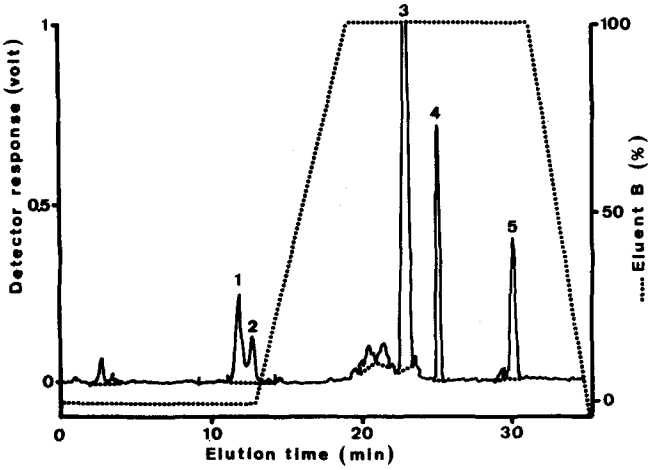


FIG. 3. Typical straight-phase high-performance liquid chromatography separation of lipids after incubation of porcine pancreatic phospholipase A_2 (PLA_2) with 254 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. Assay was performed with 10 mM $CaCl_2$, 0.03% deoxycholate in 30 mM HEPES–Tris buffer, pH 8.5, and 2.5 μg PLA_2 (final volume: 3 mL) at 37°C. Elution conditions are described in the Materials and Methods section. Peaks: 1 = oleic acid; 2 = heptadecanoic acid; 3 = deoxycholate; 4 = 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 5 = 1-oleoyl-*sn*-glycero-3-phosphocholine.

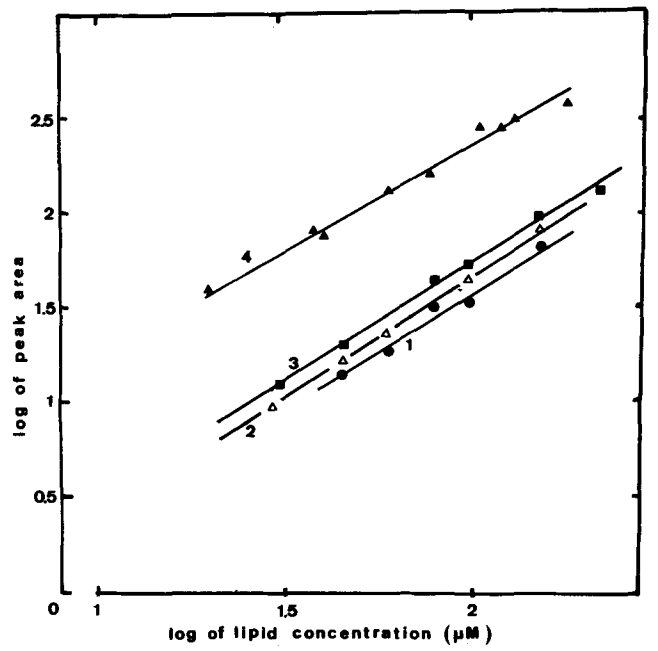


FIG. 4. Calibration curves, according to Stolyhwo *et al.* (Ref. 27), log/log plot of detector response (peak area) against concentration of lipid injected (μM). 1 (●) heptadecanoic acid, 2 (△) oleic acid, 3 (■) 1-oleoyl-*sn*-glycero-3-phosphocholine, 4 (▲) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. Stock solutions of fatty acids were in ethanol and phospholipids were emulsified in 30 mM HEPES–Tris buffer, pH 8.5, with 0.03% deoxycholate. These solutions were diluted to different concentrations in 30 mM HEPES–Tris buffer, pH 8.5, and lipids were extracted before straight-phase high-performance liquid chromatography analysis.

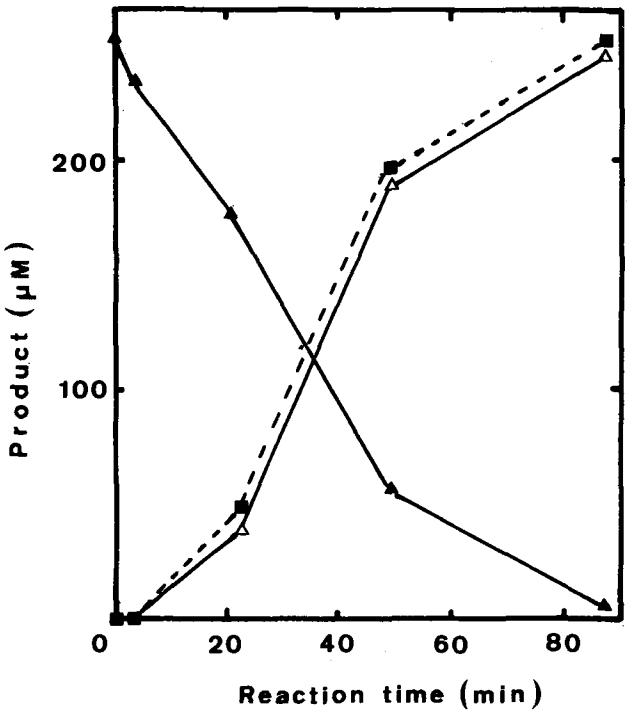


FIG. 5. Straight-phase high-performance liquid chromatography determination of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine hydrolysis, oleic acid, and 1-oleoyl-*sn*-glycero-3-phosphocholine release during incubation with porcine pancreatic PLA_2 at 37°C. Incubation conditions as in Figure 3. (▲) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; (△) oleic acid; (■) 1-oleoyl-*sn*-glycero-3-phosphocholine. See Figure 3 for abbreviation.

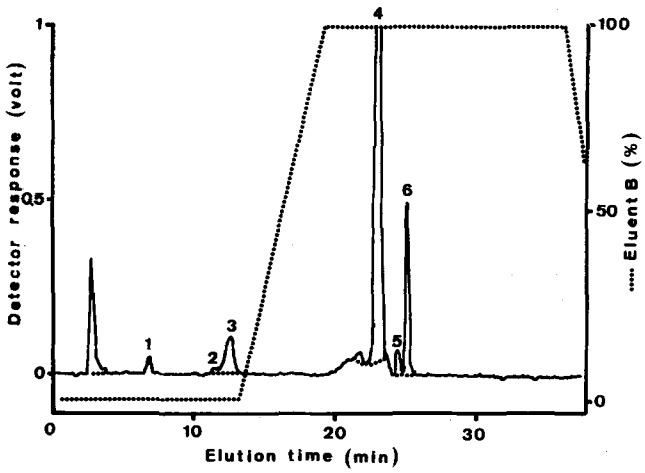


FIG. 6. Typical straight-phase high-performance liquid chromatography separation of lipids after incubation of tomato extract with 127 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. Assay was performed with 10 mM $CaCl_2$, 0.03% deoxycholate in 3 mM HEPES–Tris, pH 8.5, and 1.2 mL of tomato extract (final volume: 3 mL) at 37°C. Elution conditions are described in the Materials and Methods section. Peaks: 1 = unknown; 2 = oleic acid; 3 = heptadecanoic acid; 4 = deoxycholate; 5 = 1,2-dioleoyl-*sn*-glycero-3-phosphate; 6 = 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

Incubation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine with tomato extract gave within 110 min, and after an extraction of lipids, a typical profile as shown in Figure 6. The substrate decreased progressively with time, but only a small amount of oleic acid appeared and no 1-oleoyl-*sn*-glycero-3-phosphocholine (Fig. 7). Some 1,2-dioleoyl-*sn*-glycero-3-phosphate

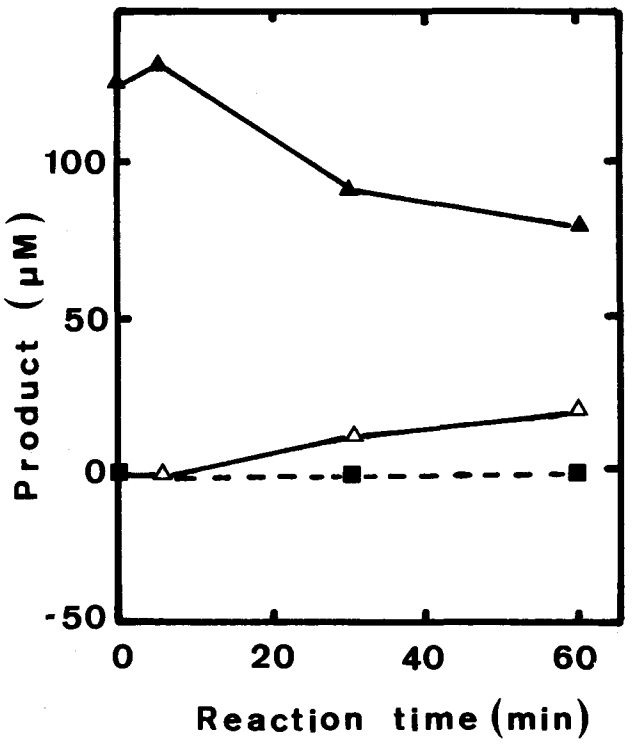


FIG. 7. Straight-phase high-performance liquid chromatography determination of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine hydrolysis and oleic acid release during incubation with tomato extract at 37°C. Incubation conditions as in Figure 6. (▲) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; (△) oleic acid; (■) 1-oleoyl-*sn*-glycero-3-phosphocholine.

TABLE 2
Comparison of Three Methods of Measurement of Phospholipase A_2 (PLA_2) Activity[a]

	Tomato extract	Porcine pancreatic PLA_2
SP–HPLC[b]	$1.7\ 10^{-4}$	1.14
Radioactivity	$1.9\ 10^{-4}$	0.95
Spectrofluorimetry	$2.6\ 10^{-4}$[c]	0.06

[a]Results are expressed in µmole of fatty acid released/min/mg protein. Experimental conditions as described in the Materials and Methods section.
[b]Straight-phase high-performance liquid chromatography.
[c]Measurement without addition of $CaCl_2$ in the reaction mixture.

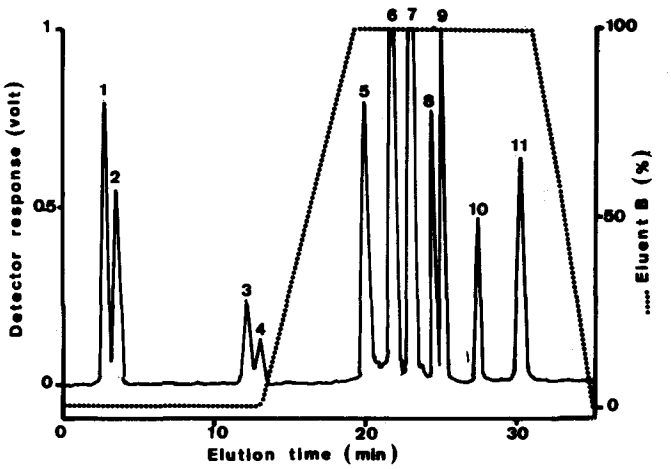


FIG. 8. Elution profile of standard lipids by straight-phase high-performance liquid chromatography (SP–HPLC). Elution conditions are described in the Materials and Methods section. Peaks: 1 = 1,2,3-trioleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, 1,3-dioleoyl-*sn*-glycerol, and oleic acid oleoyl ester; 2 = 1-oleoyl-*sn*-glycerol, 3 = oleic acid; 4 = heptadecanoic acid; 5 = 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; 6 = diphosphatidylglycerol; 7 = deoxycholate; 8 = 1,2-dioleoyl-*sn*-glycero-3-phosphate ; 9 = 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 10 = 1-oleoyl-*sn*-glycero-3-phosphate; 11 = 1-oleoyl-*sn*-glycero-3-phosphocholine. Stock solutions of glycerides and fatty acids were in ethanol and phospholipids were emulsified as in Figure 4. After dilution in 30 mM HEPES–Tris buffer, pH 8.5, lipids were extracted before SP–HPLC analysis.

also appeared (see Fig. 6, peak 5), as did an unknown product more apolar than oleic acid (see Fig. 6, peak 1). The level of 1,2-dioleoyl-*sn*-glycero-3-phosphate found by SP–HPLC was very low (peak area <20) and did not significantly change, or slightly increased, with incubation time, depending on the experiments.

Because of the unexplained low level of oleic acid liberated in comparison with the substrate consumed we checked the SP–HPLC method by the radiochemical assay, which is a currently used method. Expressed in µmole of fatty acid released/min/mg protein, the values obtained with tomato extract were approximately the same whether the measurements of phospholipase activity were made by SP–HPLC, spectrofluorimetry, or radioactivity (Table 2). Using porcine pancreatic PLA_2, results were approximately the same for SP–HPLC and radioactivity, but not for spectrofluorimetry. Moreau (9) reported that the rate of hydrolysis of NBD-phospholipids by porcine pancreatic PLA_2 is several hundredfold lower than the rates of hydrolysis of the natural phospholipids used for radiochemical and SP–HPLC methods.

In order to determine the nature of the unknown product, eight apolar lipids were dissolved in ethanol, introduced into the reaction mixture without enzyme, extracted in chloroform, and analyzed by SP–HPLC (Fig. 8). None of these apolar lipids had the retention time of the unknown product.

Substrate specificity of tomato extract. Six polar lipids were checked in order to determine retention time of potential substrates or products of phospholipases or lipases that could be present in the tomato extract. These standards were solubilized as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (see the Materials and Methods section), introduced into the reaction mixture without enzyme, extracted by the procedure of Bligh and Dyer (12), and analyzed by SP–HPLC (Fig. 8). All these phospholipids were correctly separated.

The loss of 1-oleoyl-*sn*-glycero-3-phosphocholine when 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was incubated with tomato extract could be due to hydrolysis of 1-oleoyl-*sn*-glycero-3-phosphocholine by tomato extract.

Like 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1-oleoyl-*sn*-glycero-3-phosphocholine was a substrate for tomato extract, but, as with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, the oleic acid concentration was lower than the 1-oleoyl-*sn*-glycero-3-phosphocholine consumed (Table 3). The unknown product did not appear when 1-oleoyl-*sn*-glycero-3-phospho-

TABLE 3
Hydrolytic Activity of Tomato Extract on Different Substrates Determined by SP–HPLC Assay[a]

	PC (μM)	LysoPC (μM)	LysoPA (μM)	MAG (μM)	DAG (μM)	PA (μM)
Substrate initial concentration	127	192	229	280	160	142
Consumed substrate in 30 min	34	52	229	280	0	0
Oleic acid concentration after 30 min	12	27	229	280	0	0

[a]PC = 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; LysoPC = 1-oleoyl-*sn*-glycero-3-phosphocholine; LysoPA = 1-oleoyl-*sn*-glycero-3-phosphate; MAG = 1-oleoyl-*sn*-glycerol; DAG = 1,2-dioleoyl-*sn*-glycerol; PA = 1,2-dioleoyl-*sn*-glycero-3-phosphate; SP–HPLC = straight-phase high-performance liquid chromatography. Experimental conditions as described in the Materials and Methods section.

choline was used as substrate even if the level of oleic acid was lower than the substrate consumed. There was therefore no correlation between loss of oleic acid and the presence of the unknown product. According to Reynolds *et al.* (14), PLA_2 may have lysophospholipase activity. This could explain the loss of 1-oleoyl-*sn*-glycero-3-phosphocholine after incubation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine with tomato extract, but would require that the concentration of oleic acid be twice as high as the concentration of consumed substrate, which was not the case.

Tomato extract also was able to hydrolyze 1-oleoyl-*sn*-glycero-3-phosphate and 1-oleoyl-*sn*-glycerol and, in these cases, the reaction was stoichiometric (Table 3). However, tomato extract did not accept as substrate 1,2-dioleoyl-*sn*-glycero-3-phosphate and 1,2-dioleoyl-*sn*-glycerol.

DISCUSSION

Methods based on measurement of the release of labeled (even by a fluorescent or a radioactive probe) *sn*-2 fatty acid failed to confirm that the tomato extract contained PLA_2. The release of the labeled *sn*-2 fatty acid could be accompanied by release of the *sn*-1 fatty acid without detection of this cleavage by the radioactive or the spectrofluorimetric assay. Moreover, in the spectrofluorimetric assay using C_6-NBD-PC, the fluorescence increased even when PLC released 1-acyl-2-{6[(7-nitro-2,1,3, benzoxadiazol-4-yl) amino]-caproyl}-*sn*-glycerol, after cleavage of the *sn*-3 bond (9). It is necessary to quantitate the release of 1-acyl-*sn*-glycero-3-phospholipid and free fatty acid and the decrease in substrate in order to check the stoichiometry. Nevertheless, the C_6-NBD-PC technique, which was described by Wittenauer *et al.* (7), is sensitive and useful for enzymatic activity measurements because the assay is continuous, and does not require either lipid extraction or chromatographic separations.

The variations in phospholipase activity in the fruits, which were ripened on tomato plants, could be compared to the variation in activity in ripened "cherry tomatoes" (2) and to LOX activities measured in ripened Ailsa Craig tomatoes (6). The GO stage is characterized by peaks in total phospholipids and LOX activity. LOX is known to be most active with free fatty acids as substrate, but it is also capable of specific oxygenation of fatty acids esterified in phospholipids (6,15). On the other hand, Sevanian and Kim (16) have shown that PLA_2 preferentially removes oxidized fatty acid from membrane phospholipids. On the basis of these findings, Van Kuijk *et al.* (17) proposed that a new role for PLA_2 was protection of membranes from oxidized phospholipids. After PLA_2 action on peroxidized phospholipids, the lysophospholipid produced could presumably be reacylated with a long-chain fatty acid CoA to complete the membrane, and the peroxidized fatty acid released would be reduced by the glutathione peroxidase. This suggested role of PLA_2 is in agreement with our finding that in the mutant *rin* tomato, which is unable to ripen, phospholipase activity was higher than in normal fruits.

Leshem (18) reported that in higher plants PLA_2 activity is stimulated by Ca^{2+}, whereas Hasson and Laties (19) found that lipolytic acyl hydrolase activities do not require Ca^{2+}. The phospholipase activity of tomato extract measured by spectrofluorimetry is not stimulated by 10 mM $CaCl_2$, whereas the activity of porcine pancreatic PLA_2 is strongly stimulated. The analysis of products from C_6-NBD-PC incubation with tomato extract revealed the liberation of an *sn*-2 marked fatty acid. But the absence of the correspondent 1-oleoyl-*sn*-glycero-3-phosphocholine indicates that the extract contain a mixture of phospholipases responsible for phospholipid degradation. This method cannot enable both the detection of the liberation of the fatty acid in position *sn*-1 and NBD-aminohexanoic acid. So we cannot conclude that tomato extract contains any PLA_2-type activity.

We have devised a new, one-step SP–HPLC method of separation of the products of incubation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine with porcine pancreatic PLA_2 which simultaneously quantitates the decrease in substrate and the increase in free fatty acid and 1-oleoyl-*sn*-glycero-3-phosphocholine. With this commercial enzymatic solution, it is possible to very rapidly characterize the nature of the enzyme and to check the values obtained with the spectrofluorimetric methods. Using our SP–HPLC technique, we have shown that the crude tomato extract contained some lipolytic enzymes able to hydrolyze stoichiometrically, 1-oleoyl-*sn*-glycero-3-phosphate and 1-oleoyl-*sn*-glycerol. This extract also was able to hydrolyze 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1-oleoyl-*sn*-glycero-3-phosphocholine, but in these cases, the oleic acid increase was less marked than the decrease in the substrates. In the presence of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, no 1-oleoyl-*sn*-glycero-3-phosphocholine was detectable, but an apolar product appeared as well as a small quantity of 1,2-dioleoyl-*sn*-glycero-3-phosphate in the reaction mixture.

The low level of oleic acid obtained after incubation of 1,2-dioleyl-*sn*-glycero-3-phosphocholine with tomato extract is difficult to understand. Oleic acid is not a substrate for LOX (20), so the idea of its oxidation by lipoxygenases can-

not be entertained. Other oxidations are able to transform oleic acid into epoxystearic acid, but the retention time of epoxystearic acid, measured by SP–HPLC assay, was the same as that of oleic acid. When commercial oleic acid was added to tomato extract and 1,2-dioleyl-*sn*-glycero-3-phosphocholine in the reaction mixture, no change in oleic acid was seen after one hour of incubation; there was no special activity in the tomato extract responsible for the loss of oleic acid after incubation with this substrate.

It is possible to conclude that the crude tomato extract contained phospholipase activity, as demonstrated by Vrbova *et al.* (21), and also lipolytic acyl hydrolase activity according to the definition given by Galliard (4). This author considers that deacylation of phospholipids in higher plants is catalyzed by a lipid acyl hydrolase of wide substrate specificity. A wide range of lipids is hydrolyzed: lysophospholipids and monoacylglycerols are deacylated most rapidly; phospholipids, galactolipids are also hydrolyzed, but triacylglycerols ("lipase" substrate) are not attacked.

Higher plant PLA_2 is scarcely documented. In potato, a putative PLA_2 was found, using the spectrofluorimetric method described by Wittenauer *et al.* (7), in leaves by Moreau (22), and in tubers by Kawakita *et al.* (23). The enzymatic activity was low in the membrane fraction in comparison with that found in the supernatant fraction. In potato tubers and leaves, only the soluble enzyme was shown to be Ca^{2+}-dependent. Gross *et al.* (24) and Cho and Boss (25) used the spectrofluorimetric method to determine PLA_2 activity in carrot cell cultures. Scherer and André (26) measured PLA_2 activity in the microsomal fraction of cultured soybean cells by the accumulation of radioactive 1-acyl-*sn*-glycero-3-phosphocholine hydrolyzed from radioactive 1,2-diacyl-*sn*-glycero-3-phosphocholine in membranes prelabeled with ^{14}C-choline *in vivo*. This result, obtained by a specific method, suggests there is true PLA_2 activity in higher plants and encourages us to search in the future for PLA_2 activity in purified enzymatic extract of tomato fruits.

ACKNOWLEDGMENTS

This study was carried out in the framework of contract n° CI1*-CT91-0929 "Post-harvest metabolism in plant organs and tissues" supported by the Commission for Science, Research and Development of the European Communities. The authors thank Dr. Antoine Trémolières for useful suggestions during the experiments and the writing of the text.

REFERENCES

1. Thompson, J.E., Mayak, S., Shinitzky, M., and Halevy, A.H. (1982) *Plant Physiol. 69*, 859–863.
2. Güçlü, J., Paulin, A., and Soudain, P. (1989) *Physiol. Plant. 77*, 413–419.
3. Burns, D.D., Galliard, T., and Harwood, J.L. (1980) *Phytochem. 19*, 2281–2285.
4. Galliard, T. (1979) in *Advances in the Biochemistry and Physiology of Plant Lipids* (Appelqvist, L.A., and Liljenberg, C., eds.) Elsevier, Amsterdam, pp. 121–132.
5. Moreau, R.A., and Isett, T.F. (1985) *Plant Science 40*, 95–98.
6. Droillard, M.J., Rouet-Mayer, M.A., Bureau, J.M., and Lauriere, C. (1993) *Plant Physiol. 103*, 1211–1219.
7. Wittenauer, L.A., Shirai, K., Jackson, R.L., and Johnson, J.D. (1984) *Biochem. Biophys. Res. Commun. 118*, 894–901.
8. Hendrickson, H.S., Hendrickson, E.K., and Rustad, T.J. (1987) *J. Lipid Res. 28*, 864–872.
9. Moreau, R.A. (1989) *Lipids 24*, 691–699.
10. Palmgren, M.G., Sommarin, M., Ulvskov, P., and Jorgensen, R.L. (1988) *Physiol. Plant. 74*, 11–19.
11. Vignon, E., Mathieu, P., Louisot, P., Vilamitjana, J., Harmand, M.F., and Richard, M. (1989) *J. Rheumatology 16*, 35–38.
12. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
13. Metcalfe, L.D., Schmitz, A., and Pelka, J. (1966) *Anal. Chem. 38*, 514–515.
14. Reynolds, L.J., Hughes, L.L., Louis, A.I., Kramer, R.M., and Denis, E.A. (1993) *Biochim. Biophys. Acta 1167*, 272–280.
15. Brash, A.R., Ingram, C.D., and Harris, T.M. (1987) *Biochemistry 26*, 5465–5471.
16. Sevanian, A., and Kim, E. (1985) *J. Free-Radical Biol. Med. 1*, 263–271.
17. Van Kuijk, F.J.G.M., Sevanian, A., Handelman, G.J., and Dratz, E.A. (1987) *Trends Biochem. Sci. 12*, 31–34.
18. Leshem, Y.Y. (1987) *Physiol. Plant. 69*, 551–559.
19. Hasson, E.P., and Laties, G.G. (1976) *Plant Physiol. 57*, 142–147.
20. Nicolas, J., and Drapron, R. (1981) *Sciences Aliments 1*, 91–168.
21. Vrbova, E., Kroupova, I., Valentova, O., Novotna, Z., Simond-Côte, E., Kas, J., and Thévenot, C. (1993) *6th European Congress of Biotechnology*, Firenze 13–17 June, TU 264.
22. Moreau, R.A. (1986) *Plant Sci. 47*, 1–9.
23. Kawakita, K., Senda, K., and Doke, N. (1993) *Plant Sci. 92*, 183–190.
24. Gross, W., Brglez, I., and Boss, W.F. (1991) *Plant Physiol. Supl. 96*, 447.
25. Cho, M.H., and Boss, W.F. (1992) *Plant Physiol. Supl. 99*, 718.
26. Scherer, G.F.E., and André, B. (1993) *Planta 191*, 515–523.
27. Stolyhwo, A., Martin, M., and Guiochon, G. (1987) *J. Liq. Chrom.*, 1237–1253.

[Received September 15, 1994; in revised form May 29, 1995; Revision accepted June 7, 1995]

Substrate Specificity of the Lipase from *Candida parapsilosis*

Delphine Briand[a], Eric Dubreucq[a,*], Jean Grimaud[b] and Pierre Galzy[a]

[a]Ecole Nationale Supérieure Agronomique, Institut National de la Recherche Agronomique, 34060 Montpellier, France and [b]Ecole Nationale Supérieure de Chimie, 34053 Montpellier, France

ABSTRACT: Substrate specificity of the acyltransferase activity of the lipase (EC 3.1.1.3) from *Candida parapsilosis* CBS 604 was studied in aqueous media. The specificity toward both acid and alcohol parts of a large number of acylglycerols and aliphatic esters was investigated. This lipase showed a high activity in the presence of esters with long-chain fatty acids and particularly unsaturated fatty acids with a *cis*-Δ9 double bond. It was observed that the activity profile depended not only on the alcohol part of the acyl ester, but also on the temperature of the reactant medium. The best lipid substrates had their melting point between −40 to +20°C, 14 to 18 carbon atoms in the acyl group and 1 to 4 carbon atoms in the alkyl group. The enzyme, defined as an acyltransferase in a previous paper, showed a high affinity for primary and secondary alcohols with a short carbon chain (1 to 5 carbon atoms) as acyl acceptors. The influence of free alcohols in the reactant medium on the hydrolysis and alcoholysis activities of the enzyme is discussed. Two phenomena seem to be involved, depending on the alcohol: competition with water for the acyltransfer reaction and lipid substrate dilution when the alcohol places at the oil/water interface.
Lipids 30, 747–754 (1995).

Lipases (EC 3.1.1.3) are characterized by their ability to catalyze the rapid hydrolysis of ester bonds at the interface between an insoluble substrate phase (triacylglycerols) and an aqueous phase in which the enzyme is dissolved. Lipases are sometimes able to operate in poorly hydrated organic media in which lipids are soluble. Under such conditions, numerous lipases catalyze reactions such as alcoholysis, transesterification, and synthesis of esters of fatty acid (FA). This process has many advantages over conventional chemical catalysis for the production of peculiar esters. In particular, the reaction is performed under mild conditions which prevent the polymerization of polyunsaturated FA (1). Besides, reactions catalyzed by lipases are often highly specific. As lipases react in a heterogeneous system, the substrate specificity may depend not only on the chemical structure of substrate molecules but also on the physical properties of the emulsion. The numerous reports on the specificity of lipases generally focus on the influence of the chemical structure (notably FA chain length and unsaturation and FA location in the glycerides) and of the chiral properties of the substrates, but do not take into consideration their physical properties. Nevertheless, it has been shown that the surface pressure of substrate monolayers is a factor affecting the rate of hydrolysis catalyzed by pancreatic lipase (2). It has also been found that the activity spectrum of the lipase from *Chromobacterium viscosum* varies with the reactant medium temperature (3).

In recent publications, we have shown and described the acyltransfer activity of the lipase from *Candida parapsilosis* in aqueous media (4,5). In the presence of methanol in the aqueous phase, methyl esters were produced by alcoholysis of triacylglycerols, and, under optimal conditions, hydrolysis of the substrates and the products was almost completely inhibited. Hydrolysis was not reversible under the reaction conditions used. The present paper describes the substrate specificity of this lipase in aqueous media. We have studied the importance of the nature of both acid and alcohol parts of the substrates with attention to their physicochemical properties.

MATERIALS AND METHODS

Organism. The strain used was *C. parapsilosis* (Ashford, Langeron, and Talice), CBS 604, obtained from the Centraalbureau voor Schimmelcultures, Yeast division (Delft, Holland).

Culture techniques. All cultures were performed in Erlenmeyer flasks filled to 10% of their volume, incubated at 28°C, and shaken at 80 oscillations min^{-1} (amplitude 7 cm). The basal medium was medium G (6) adjusted to pH 6.5 with a 50 mM NaH_2PO_4/Na_2HPO_4 phosphate buffer. The carbon source was rapeseed oil (0.5% wt/vol) sterilized by autoclaving at 120°C for 20 min.

Enzyme extract and substrates preparation. The culture was defatted three times with hexane (vol/vol) at the end of the exponential phase. The cells were then harvested by centrifugation (7000 × *g*, 15 min). The supernatant contained the enzyme, which was then purified according to Riaublanc *et al.* (7). The lipid substrates were emulsified (5% wt/vol) in an aqueous poly(vinyl alcohol) solution (2% wt/vol) by sonication as described by Biehn and Ernsberger (8).

Analytical techniques: (i) Gas chromatography (GC). Short-chain FA and long-chain FA methyl esters were sepa-

*To whom correspondence should be addressed at UFR de Microbiologie Industrielle et de Génétique des Microorganismes, 2 Place Viala, 34060 Montpellier Cedex 1, France.

Abbreviations: FA, fatty acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

rated using a fused apolar capillary column coated with DB23 (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific, Folsom, CA). Temperatures of the injector and of the flame-ionization detector were 200 and 250°C, respectively. For short-chain FA, the column oven was kept at 80°C for 1 min then heated up to 120°C (1°C · min^{-1}), while for long-chain FA methyl esters the column oven was kept at 140°C for 1 min then heated up to 200°C following a temperature gradient of 2.5°C min^{-1}.

(ii) High-performance liquid chromatography (HPLC). The column used was a Spherisorb ODS2 (250 × 4.6 mm, particule size 5 μm; Alltech Associates, Inc., Deerfield, IL) thermostated at 30°C. Absorbance was read at 210 nm. The solvent was acetonitrile/acetone (1:1, vol/vol). The flow rate was maintained at 0.8 mL/min for 6 min, then increased from 0.8 to 2 mL/min in 4 min, maintained at 2 mL/min for 22 min, and finally decreased to 0.8 mL/min in 4 min.

(iii) Thin-layer chromatography (TLC). Migration was carried out on silica 60G (20 × 10 cm × 0.25 mm; Merck, Darmstadt, Germany) using a hexane/diethyl ether/acetic acid mixture (70:30:1, by vol). The plates were dried and sprayed with a copper acetate/orthophosphoric acid mixture (1:1, vol/vol) before charring at 180°C for 10 min to visualize the sample bands.

(iv) Melting point determination. Melting points were determined using a Mettler FP5 apparatus, equipped with an FP51 cell (Mettler instrumente AG, Zürich, Switzerland).

Enzyme assays: (i) Standard reaction conditions. An emulsion of lipid substrate (200 μL) was added to 2 mL of a 50 mM NaH_2PO_4/Na_2HPO_4 phosphate buffer, pH 6.5, containing the purified enzyme (0.16 U) and the alcohol of interest at various concentrations. The reaction was carried out at 45°C for 15 min and was stopped by the addition of an ethanol/acetone/sulfuric acid (50:50:0.1, by vol) mixture. Long-chain acids or esters were extracted with hexane. Acetic, butyric, and propionic acids and methyl esters were determined in the aqueous phase. One enzyme unit (U) is the quantity of enzyme that releases 1 μmol FA from rapeseed oil in one minute in the standard conditions, in the absence of alcohol. Each experiment was done at least in triplicate and the results were expressed as the mean of three determinations. The highest error on mean was 15%.

(ii) Analysis of the products. Determination of total long-chain free FA was performed according to Van Autryve *et al.* (9). Short-chain free FA were quantified by GC. When required, long-chain free FA were first separated from the other components of the reaction medium by TLC, scraped off the plate, and then methylated. One mL of 12.5% boron trifluoride in methanol was added to the FA in a sealed ampule. This solution was heated to 100°C for 2 min in a water bath. The FA methyl esters were then extracted with hexane after the addition of 1 mL of water. Methyl esters, dissolved in acetone, were analyzed by HPLC.

Reagents. The esters studied (>99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO) or Nu-Chek-Prep, Inc. (Elysian, MN). All other reagents were at least of analytical grade.

RESULTS

The bioconversion of rapeseed oil in the presence of the lipase from *C. parapsilosis* and 2.2 M methanol was followed for 8 h in the standard reaction conditions. The FA of the triacylglycerols of rapeseed oil were converted into methyl esters (53%) and free FA (11.5%). These products were separated by TLC, and their FA composition determined (Table 1). Relative frequencies of the main FA were different in oil, in the methyl esters formed by alcoholysis, and in the free FA released by the residual hydrolysis reaction. Noticeably, palmitic and stearic acids were concentrated in the free FA and almost totally absent in methyl esters. This preliminary experiment indicated that hydrolysis and methanolysis do not apparently show the same selectivity. A deeper study of the substrate specificity of the enzyme was thus undertaken.

Acyl donor specificity. Hydrolysis of various monoesters was performed under the standard conditions. The respective melting points of the substrates tested are given with the results in Table 2. Results are expressed relatively to the rate of hydrolysis of rapeseed oil (0.16 μmol free FA released in one minute in the presence of 0.16 U). The melting point data will be discussed later.

The enzyme displayed a large activity spectrum, including esters of short carbon chain alcohols, nitrophenol, sorbitol, and fatty alcohols. Methyl ester hydrolysis rates showed important variations with the nature of the acyl group. Esters of long-chain FA (more 12 carbon atoms) were the favored substrates. Monounsaturated methyl esters with a *cis*-Δ9 double bond were more rapidly hydrolyzed than both their *cis*-Δ6 and *cis*-Δ11 isomers and their saturated counterpart.

Figure 1 displays activity vs. FA chain-length profiles for aliphatic methyl esters. The graph shows a clear preference for long-chain FA with 14 to 18 carbon atoms. The profile was the same for *cis*-Δ9 monounsaturated and saturated FA, whereas monounsaturated esters were hydrolyzed at a higher rate. On the other hand, the rate of hydrolysis of oleic and lauric esters of aliphatic alcohols decreased with increasing length of the alcohol chain (Fig. 2).

Hydrolysis and methanolysis of homogeneous acylglycerols were then studied. Methanol concentration was 2.2 M, when present. The bioconversion of rapeseed oil, catalyzed by 0.16 U of the enzyme and corresponding to a relative activity of 100%, released 0.16 μmol free FA in one minute in

TABLE 1
Fatty Acid Composition of Free Fatty Acids and Methyl Esters Formed by Bioconversion of Rapeseed Oil in the Presence of 2.2 M Methanol[a]

Major fatty acids in rapeseed oil (94% of total free fatty acids)	Initial rapeseed oil substrate (%)	Methyl esters (%)	Free fatty acids (%)
Oleic acid	61.0	69.0	39.5
Linoleic and linolenic acids	32.0	31.0	7.5
Palmitic acid	6.0	1.0	42.0
Stearic acid	2.0	0.0	11.0

[a]The equilibrium state was obtained after 8 h in the standard conditions.

TABLE 2
Hydrolysis Activity Measured on Various Monoesters

Substrate	Hydrolysis activity[a] (%)	Melting point (°C)
Reference: rapeseed oil	100	<15[d]
Acetic acid oleyl ester	0	<15[d]
Acetic acid lauryl ester	0	<15[d]
Propionic acid ethyl ester	0	−63[b]
Caproic acid methyl ester	25	−71[b]
Heptanoic acid methyl ester	10	−56[b]
Undecanoic acid methyl ester	12	<15[d]
Undecylenic acid methyl ester	32	−28[b]
Lauric acid methyl ester	22	5[b]
Lauric acid lauryl ester	10	27.9[c]
Lauric acid nitrophenyl ester	221	47.1[c]
Lauric acid cholesteryl ester	8	80.5[c]
Lauric acid oleyl ester	0	<15[d]
Myristic acid methyl ester	36	19[b]
Myristoleic acid methyl ester (*cis*-9)	53	−4[b]
Palmitic acid methyl ester	54	30[b]
Palmitoleic acid methyl ester (*cis*-9)	133	5[b]
Stearic acid methyl ester	42	39.1[b]
Oleic acid methyl ester (*cis*-9)	113	19.9[b]
Oleic acid ethyl ester	120	<15[d]
Oleic acid propanyl-1ester	55	<15[d]
Oleic acid butanyl-1 ester	97	<−10[b]
Oleic acid lauryl ester	11	<15[d]
Oleic acid oleyl ester	21	<15[d]
Monooleic acid sorbitol ester	55	<15[d]
Vaccenic acid methyl ester (*cis*-11)	32	<15[d]
Petroselinic acid methyl ester (*cis*-6)	1	<15[d]
Linoleic acid methyl ester (*cis*-9,12)	112	−35[b]
α-Linolenic acid methyl ester (*cis*-9,12,15)	78	−57[b]
γ-Linolenic acid methyl ester (*cis*-6,9,12)	43	<15[d]

[a]Results are expressed as percentages of the reference activity determined in the standard conditions on rapeseed oil.
[b]*Handbook of Chemistry and Physics* (Ref. 10).
[c]Melting points determined experimentally.
[d]Melting points which could not be determined with the method used, compounds liquid at 15°C.

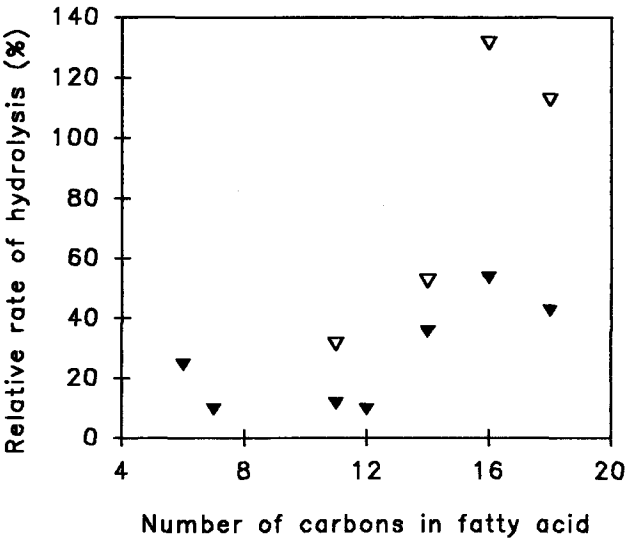


FIG. 1. Effect of the acyl chain length on the hydrolysis activity of various methyl esters. Results are expressed as percentages of the reference activity determined in the standard conditions on rapeseed oil; (▼) saturated methyl esters, (▽) *cis*-Δ9 monounsaturated methyl esters.

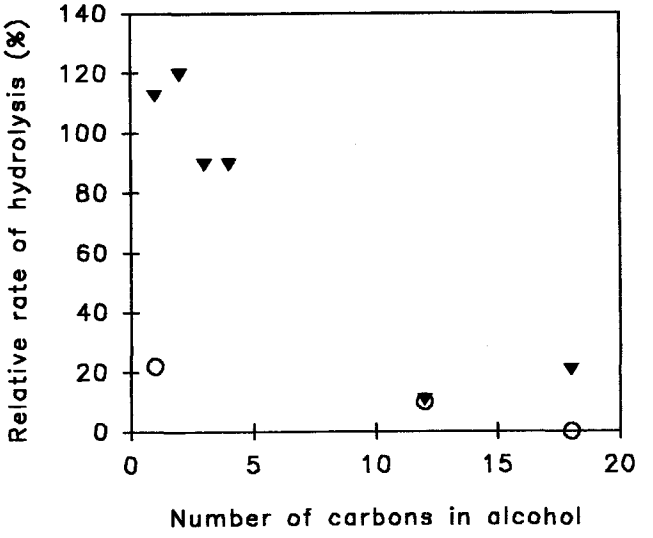


FIG. 2. Effect of the alcohol chain length on the hydrolysis activity of various esters of oleic (▼) and lauric (○) acids. Results are expressed as percentages of the reference activity determined in the standard conditions on rapeseed oil.

the absence of methanol and 0.18 μmol methyl esters in the presence of 2.2 M methanol (Table 3). A number of triacylglycerols were not or very slowly hydrolyzed. Among them were those containing saturated, Δ6-unsaturated, or *trans*-unsaturated acyl groups. Water-soluble tributyrin was also unreactive. Trilinolenin was the best substrate. It is interesting to observe that trilinolein was hydrolyzed at a higher rate than rapeseed oil, whereas it was methanolyzed at a much lower rate. This confirms the differences of selectivity of hydrolysis and methanolysis suggested by the bioconversion of rapeseed oil in the presence of methanol. It is also noticeable that, while 1,3-diolein and 1,2-diolein were hydrolyzed at the same rate, methanolysis of 1,2-diolein was more than two times as slow. This last observation is consistent with the description of the functioning of this enzyme that we have recently published (5), i.e., a 1(3)-regioselectivity—not a strict regiospecificity—of the enzyme, which is masked in the absence of a free alcohol, like methanol, due to a high rate of acyltransfer between acylglycerols; when methanol is present in the reactant medium, it competes with the free hydroxyl groups of the acylglycerols for the acyltransfer reaction, thus revealing the true regioselectivity of the enzyme.

A comparison of the hydrolysis of methyl esters and homogeneous triacylglycerols containing the same acyl groups showed differences in the activity spectrum according to the esterified alcohol (Tables 2 and 3). Saturated palmitic and stearic acid methyl esters were relatively well hydrolyzed (about half the reference activity) contrary to the corresponding triacylglycerols, which were not transformed. More curiously, hydrolysis rates of the esters of oleic, linoleic, and α-linolenic acids increased with the number of double bonds

TABLE 3
Hydrolysis and Methanolysis Activities Measured on Homogeneous Acylglycerols

Substrate	Hydrolysis activity[a] (%)	Methanolysis activity[a] (%)	Melting point (°C)
Tributyrin	0	0	<–75[b]
Trilaurin	2	2	49[b]
Tripalmitin	0	0	66.4[b]
Tripalmitolein (*cis*-9)	107	130	<15[d]
Tristearin	0	0	78.5[b]
Triolein (*cis*-9)	126	65	5[b]
Trielaidin (*trans*-9)	0	0	41[b]
Trivaccenin (*cis*-11)	37	42	<15[d]
Tripetroelaidin (*trans*-6)	0	0	52[b]
Tripetroselinin (*cis*-6)	17	7	28[b]
Trilinolein (*cis*-9,12)	140	37	–11[b]
Trilinolenin (*cis*-9,12,15)	163	170	–24[b]
Triarachidonin (*cis*-6,9,12,15)	6	0	<15[d]
Trieicosenoin (*cis*-11)	4	0	20.8[c]
Trierucin (*cis*-13)	0	0	32[b]
1,3-Diolein	70	78	26–28[b]
1,2-Diolein	70	26	<15[d]
1-Monoolein	25	28	35[b]

[a]Results are expressed as percentages of the reference activities on rapeseed oil determined in the standard conditions in the presence or absence of 2.2 M methanol.
[b]*The Lipid Handbook* (Ref. 11).
[c]Melting point determined experimentally.
[d]Melting points which could not be determined with the method used, compounds liquid at 15°C.

when the alcohol part was glycerol, whereas it decreased when the esterified alcohol was methanol.

In another experiment, we have studied the hydrolysis and the methanolysis of triacylglycerols containing two palmitic and one oleic acyl groups, the latter being either on the position 1(3)- or 2- of the glycerol molecule. The results, reported in Table 4, show that: (i) 1,2-dipalmitoyl-3-oleoylglycerol is a much better substrate of the enzyme than the other positional isomer for both reactions; (ii) 1,3-dipalmitoyl-2-oleoylglycerol is a much better substrate for hydrolysis than methanolysis. More free palmitic acid than palmitic acid methyl ester was formed from this substrate. As saturated FAs are generally located on positions 1 and 3 of triacylglycerols of vegetable oils, this result explains the accumulation of free palmitic acid during the bioconversion of rapeseed oil in the presence of methanol; (iii) the location of the FA on the glycerol molecule influences both the rate of the reaction and the FA composition of the products formed.

Acyl acceptor specificity. Alcoholysis of triolein in the presence of increasing aliphatic alcohol (ranging from methanol to butanol) concentrations was analyzed in a previous work (5) in order to understand the functioning of the lipase from *C. parapsilosis* in terms of regioselectivity. In the present study, the determination of the influence of the nature and the concentration of the acyl acceptor was extended to pentanol and branched alcohols; in order to simplify the analysis of the reactant medium, the acyl donors used were oleic acid ethyl ester and triolein.

Figure 3 shows the influence of increasing alcohol concentrations on the rate of hydrolysis of oleic acid ethyl ester. The alcohols studied were of various classes, chain length (one to five carbon atoms), and some of them were branched. The intensity of the inhibition depended on the alcohol tested. For a similar concentration, inhibition by primary alcohols increased with their carbon chain length [Fig. 3 (A and B)]. For a similar carbon chain length and a similar concentration, primary alcohols were more inhibiting than secondary alcohols [Fig. 3 (C and D)]. For a similar number of carbon atoms, branched chain alcohols reduced less hydrolysis than their aliphatic counterparts (Fig. 3E). Similar results were obtained using triolein or rapeseed oil as lipid substrate, the only difference being that their hydrolysis was less inhibited than oleic acid ethyl ester hydrolysis.

The esters formed were determined by HPLC. The alcohol concentrations giving the highest quantity of esters (optimal concentration) from oleic acid ethyl ester and triolein are given in Table 5. At these concentrations, hydrolysis was strongly inhibited. For higher concentrations, both hydrolysis and alcoholysis were inhibited. Esters formed with all the alcohols tested except for tertiary butanol. The absence of affinity toward tertiary alcohols seems to be general among lipases (12,13). Although the optimal alcohol concentration decreased significantly with increasing carbon chain length of the alcohol in both cases, comparable amounts of esters formed from oleic acid ethyl ester and alcohols from methanol to butanol at the optimum, whereas methanol appeared to be the privileged substrate when the acyl donor was triolein. The amount of esters formed thus depended on the nature of both the acyl acceptor (alcohol) and the acyl donor (oleic acid ethyl ester or triolein). It is to be noted that tertiary butanol, even though not a substrate of the enzyme for the al-

TABLE 4
Hydrolysis and Methanolysis of Positional Isomers of Heterogeneous Triacylglycerols

Substrate	Relative rate of hydrolysis[a] (%)	Free fatty acid composition (%)		Relative rate of methanolysis[a] (%)	Methyl esters composition (%)	
		$C_{16:0}$	$C_{18:1}$		$C_{16:0}$	$C_{18:1}$
1,3-Dipalmitoyl-2-oleoyl glycerol	32	52	48	9	70	30
1,2-Dipalmitoyl-3-oleoyl glycerol	74	58	42	54	58	42

[a]Results are expressed as percentages of the reference activities on rapeseed oil determined in the standard conditions in the presence or absence of 2.2 M methanol.

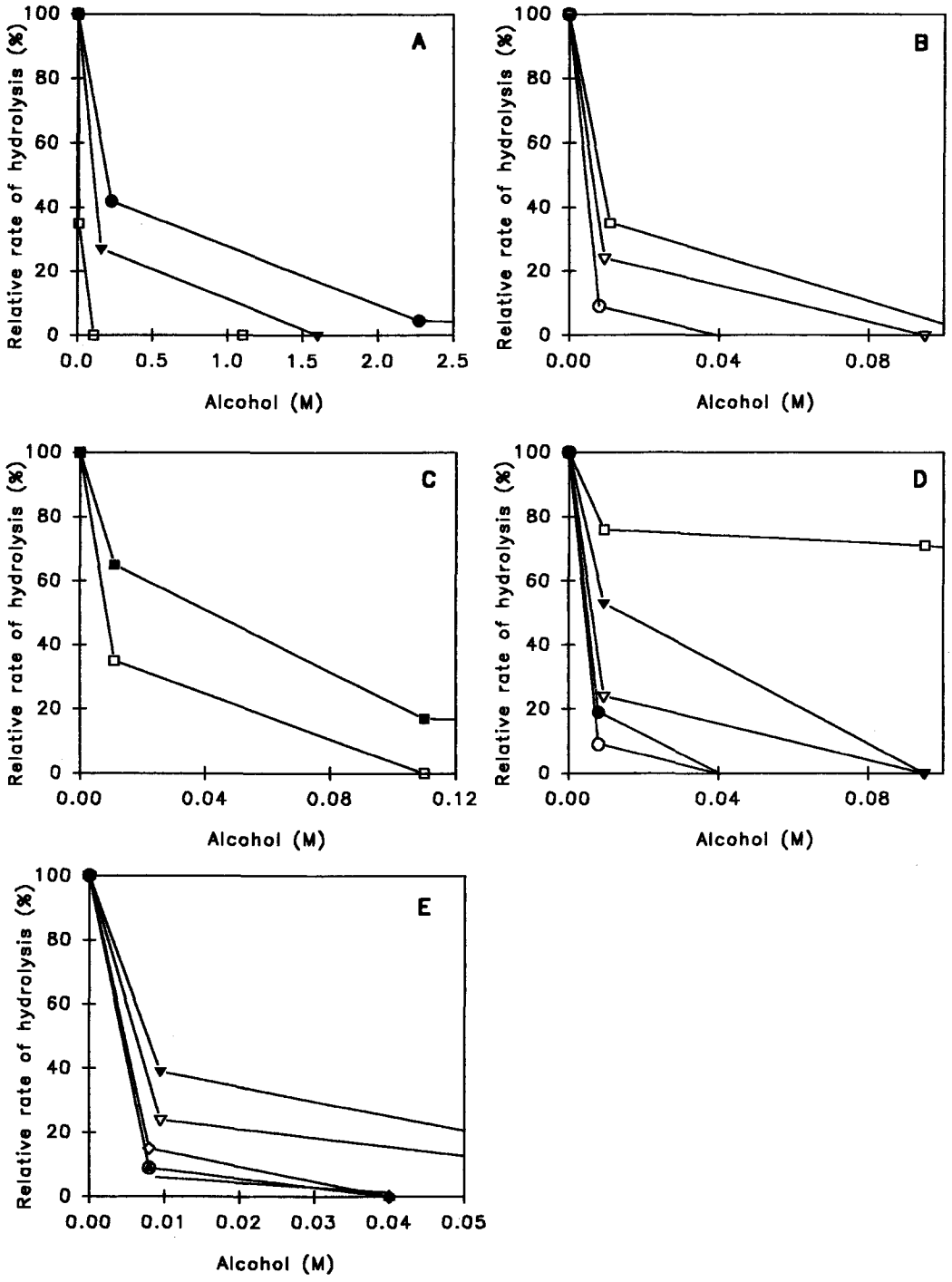


FIG. 3. Residual hydrolysis of oleic acid ethyl ester in the presence of various alcohols in the reactant medium. Results are expressed as percentages of the reference activity determined in the standard conditions without alcohol; A: (●) methanol, (▼) ethanol, (□) 1-propanol; B: (□) 1-propanol, (▽) 1-butanol, (○) 1-pentanol; C: (□) 1-propanol, (■) 2-propanol; D (▽) 1-butanol, (▼) 2-butanol, (□) *tert*-butanol, (○) 1-pentanol, (●) 3-pentanol; E (▽) 1-butanol, (▼) 2-methyl-1-propanol, (○) 1-pentanol, (◇) 3-methyl-1-butanol; (▲) 2-methyl-1-butanol.

coholysis reaction, was an inhibitor of both the hydrolysis and the methanolysis reactions (Table 6). Inhibition of both reactions was of the same level for each tertiary butanol concentration tested, indicating an inhibition of the overall acyltransfer activity.

Influence of temperature. It is well known that in the temperature range compatible with lipase activity, physical properties of lipids are very versatile and are dramatically influenced by parameters like the conformation and the location of double bonds, or the nature and the position of acyl groups

TABLE 5
Optimal Alcohol Concentrations for the Ester Formation from Oleic Acid Ethyl Ester and Triolein

Alcohol (acyl acceptor)	Optimal concentration (M)	Esters formed from oleic acid ethyl ester (acyl donor) (μmol)	Esters formed from triolein (acyl donor) (μmol)
Mehanol	2.270	3.00	2.18
Ethanol	1.600[a]	—	0.46
1-Propanol	0.100	2.85	0.56
2-Propanol	0.100	2.85	0.29
1-Butanol	0.010	2.25	0.60
2-Butanol	0.100	1.95	0.44
2-Methyl-1-propanol	0.010	0.90	n.d.[c]
Tertiary butanol[b]	—	—	—
1-Pentanol	0.008	0.45	n.d.
3-Pentanol	0.008	0.90	n.d.
2-Methyl-1-butanol	0.008	0.90	n.d.
3-Methyl-1-butanol	0.008	1.20	n.d.

[a]Determined in the presence of triolein only.
[b]Alcoholysis was not observed.
[c]n.d., Not determined.

TABLE 6
Residual Hydrolysis and Methanolysis of Triolein in the Presence of Increasing Tertiary Butanol Concentrations[a]

Tertiary-butanol concentration (M)	Hydrolysis activity[b] (%)	Methanolysis activity[c] (%)
0.000	100	100
0.010	100	93
0.045	97	87
0.090	95	80
0.220	75	68
0.450	61	64
0.900	27	13

[a]Results are expressed as percentages of the reference activities on triolein determined in the standard conditions in the absence[b] or in the presence[c] of 2.2 M methanol.

on the acylglycerols. As the susbtrate specificity of the enzyme was difficult to interpret, we wanted to check whether the physical properties of the substrates (particularly their melting point) were as important for the activity as was their chemical structure. This study was focused on hydrolysis reaction because of the numerous substrates available (Tables 2 and 3). In a general manner, the substrates with a melting point higher than 40°C were very slowly hydrolyzed. The melting point of the preferential substrates was contained between −40 and +20°C.

As temperature influences the physical properties of lipids, its effect on the hydrolysis rate of several saturated (Fig. 4A) and monounsaturated (Fig. 4B) methyl esters was examined. The results showed that whereas palmitic and palmitoleic methyl esters were the best substrates at 35 and 45°C, the activity spectrum tended to be shifted toward shorter chain FA with increasing temperatures. Hydrolysis rates of lauric, myristic, and myristoleic methyl esters increased with temperature contrary to the longer chain esters, for which hydrolysis activity was maximal at 45°C. The rate of hydrolysis of myristoleic acid methyl ester was more than doubled between 35 and 55°C, while it was reduced by one third and more than a half for palmitoleic and oleic acid methyl esters, respectively. The activity spectrum of the lipase thus varied with the temperature of the reactant medium.

DISCUSSION

The influence of the nature of the fatty acyl and alcohol parts of various esters on the hydrolysis and alcoholysis reactions, catalyzed in aqueous medium by the acyltransferase from *C. parapsilosis*, was investigated. The influence of alcohols likely to be acyl acceptors in the alcoholysis reaction was also studied. Complex results were obtained concerning the FA selectivity of the enzyme, depending on the esterified alcohol, the location of the FA on the glycerol molecule in the case of triacylglycerols, and the reaction conditions. Nevertheless, we showed that long-chain *cis*-Δ9 unsaturated FA esterified with short-chain alcohols were good acyl donor substrates and that short chain alcohols were good acyl acceptors.

Physical properties of the acylglycerols are very dependent on the esterified FA. For example, triacylglycerols of long-chain saturated and *trans*-unsaturated FA are solid at the temperature of the assay. The low activity of the enzyme on these substrates is thus likely to be largely due to their physical state. As emphasized by Jensen *et al.* (14), the melting point has a major influence on substrate emulsifiability. A slow rate of hydrolysis of solid triacylglycerols is a general feature of microbial lipases (15).

Temperature was shown to influence the activity spectrum of the enzyme. This also had been described in the case of a lipase from *Chr. viscosum* (3). However, the activity spectrum of the lipase from *Chr. viscosum* was shifted toward long-

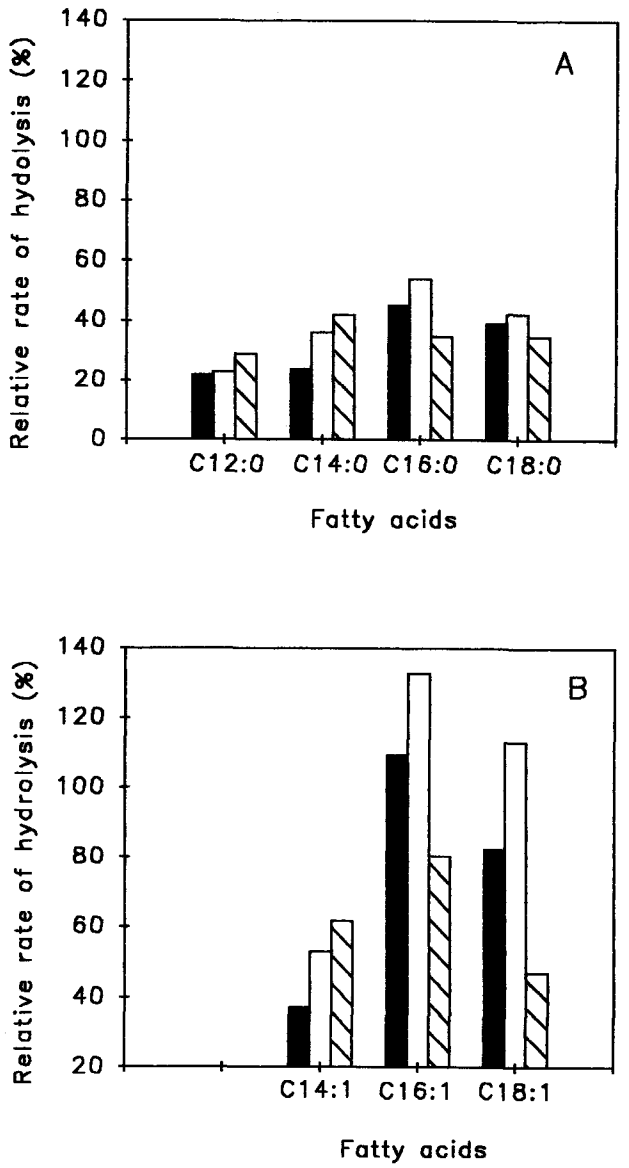


FIG. 4. Effect of the reactant temperature on the activity profile. Results are expressed as percentages of the reference activity determined in the standard conditions on rapeseed oil. 4A: Saturated methyl esters (■) 35°C, (□) 45°C, (striped bar) 55°C; B: *cis*-Δ9 monounsaturated methyl esters (■) 35°C, (□) 45°C, (striped bar) 55°C.

chain (i.e., more than 16 carbon atoms) FA when the temperature increased, whereas medium-chain (12 and 14 carbon atoms) FA were favored in the presence of the lipase from *C. parapsilosis*. A raise in the temperature of the reactant medium, by lowering the viscosity of the lipids (especially when these are saturated), will favorably modify their emulsion state. This was the explanation suggested in the case of the lipase from *Chr. viscosum*. Other phenomenons certainly superimpose in the case of the lipase of *C. parapsilosis*, as suggested by the fact that hydrolysis of palmitic and stearic esters was slower at temperatures which enhanced hydrolysis of lauric and myristic acid esters. Conformation changes of the enzyme may be involved. According to the concept of "induced fit" (16), only some substrates could release the energy required for the change in the conformation of lipases to forms which would be much more efficient catalysts. Substrates which are too small or possess too few double bonds would not be able to release enough energy. In such cases, the changes in conformation would be incomplete and optimal activity could not be achieved. A part of the energy required for the desired conformational change could be brought by thermal agitation. The state of the emulsion, and thus the quality of the interface between the enzyme and its lipid substrates, is also very dependent on the physicochemical conditions of the reactant medium and on the properties (rheology, solubility, hydrophobicity) of the substrates.

The interpretation of the influence of acyl acceptor alcohols is also complex because these compounds are not only substrates of the alcoholysis reaction but also have solvent properties which are physicochemical factors influencing the activity. They solubilize lipids and might change the lipase conformation by favoring the location of the hydrophobic groups of amino acids at the periphery of the proteins (17). The inhibition of hydrolysis by alcohols also involves several phenomena. Primary and secondary alcohols have been shown to compete with water for the acyltransfer reaction, thus inhibiting hydrolysis, in the case of the lipase of *C. parapsilosis* in aqueous medium. For alcohol concentrations higher than the optima given in Table 5, an overall inhibition effect of both hydrolysis and alcoholysis was observed. Moreover, an inhibition of the mass transfer of the lipid substrate at the reactant interface (interfacial inhibition) also has been described for various aliphatic alcohols used with pancreatic lipase (18); alcohols tend to be located at the interface, thus preventing the lipid substrate from accessing to the active area. In this case, the reaction rate was thus reduced by substrate dilution and not by competition. The inhibition effect was total when the alcohol occupied the interfacial area completely. As in our study, the less hydrophilic alcohols, i.e., the longest carbon chain alcohols, were shown to be the strongest inhibitors of hydrolysis by the pancreatic lipase.

The interfacial inhibition by alcohols certainly adds to the competition between alcohols and water in the case of the lipase from *C. parapsilosis*. Water/alcohol competition inhibits hydrolysis in favor of alcoholysis, whereas both reactions are reduced by interfacial inhibition. In the case of the short chain, very water-soluble, methanol, interfacial inhibition is negligible and competition with water is probably the main effect. In the case of longer chain alcohols, which are much less hydrophilic, the interfacial inhibition rapidly becomes the main effect with increasing alcohol concentrations. In the case of tertiary butanol, which was not a substrate of the enzyme for the alcoholysis reaction but inhibited the overall acyltransferase activity, interfacial inhibition seems to be the only effect.

The effects of substrates on the catalytic activities of the lipase of *C. parapsilosis* in aqueous medium are thus difficult to interpret in strict terms of affinity or specificity. Nevertheless, the enzyme has been shown to be able to catalyze the hy-

drolysis and the alcoholysis of a large spectrum of esters in the presence of various alcohols.

REFERENCES

1. Van der Paadt, A., Keurentjes, J.T.F., Sewalt, J.J.W., van Dam, E.M., van Dorp, L.J., and van't Riet, K. (1992) Enzymatic Synthesis of Monoglycerides in a Membrane Bioreactor with an Inline Adsorption Column, *J. Am. Oil Chem. Soc. 69*, 748–754.
2. Tavridou, A., Avranas, A., and Arzoglou, P. (1992) A Mathematical Approach to Lipolysis Based on the Relationship of Physicochemical and Biochemical Data, *Biochem. Biophys. Res. Comm. 186*, 746–752.
3. Sugiura, M., and Isobe, M. (1975) Studies on the Lipase of *Chromobacterium viscosum* IV. Substrate Specificity of a Low Molecular Weight Lipase, *Chem. Pharm. Bull. 23*, 1226–1230.
4. Briand, D., Dubreucq, E., and Galzy P. (1994) Enzymatic Fatty Esters Synthesis in Aqueous Medium with Lipase from *Candida parapsilosis* (Ashford) Langeron and Talice, *Biotechnol. Lett. 16*, 813–818.
5. Briand, D., Dubreucq, E., and Galzy P. (1995) Functioning and Regioselectivity of the Lipase of *Candida parapsilosis* (Ashford) Langeron and Talice in Aqueous Medium. New Interpretation of Regioselectivity Taking Acyl Migration into Account, *Eur. J. Biochem. 228*, 169–175.
6. Galzy, P. (1964) Etude Génétique et Physiologique du Métabolisme de l'Acide Lactique Chez *Saccharomyces cerevisiae* Hansen, *Ann. Technol. Agric. 13*, 109–259.
7. Riaublanc, A., Ratomahenina, R., Galzy, P., and Nicolas, M. (1993) Peculiar Properties of Lipase from *Candida parapsilosis* (Ashford) Langeron and Talice, *J. Am. Oil Chem. Soc. 70*, 497–500.
8. Biehn, G.F., and Ernsberger, M.L. (1948) Polyvinyl Alcohol as an Emulsifying Agent, *Ind. Eng. Chem. 40*, 1449–1453.
9. Van Autryve, P., Ratomahenina, R., Riaublanc, A., Mitrani, C., Graille, J., and Galzy, P. (1991) Spectrophotometry Assay of Lipase Activity Using Rhodamine 6G, *Oléagineux 46*, 29–31.
10. Weast, R.C. (1965–1966) *Handbook of Chemistry and Physics*, 46th edn., The Chemical Rubber Co., Cleveland.
11. Gunstone, F.D., Harwood, J.L., and Padley, F.B. (1986) *The Lipid Handbook*, 1st edn., Chapman and Hall, London, New York.
12. Légier, V., and Comeau, C. (1992) Continuous Synthesis of Esters by Cell-Bound Fungal Lipases in an Organic Solvent, *Appl. Microbiol. Biotechnol. 37*, 732–736.
13. Okumura, S., Iwai, M., and Tsujisaka, Y. (1979) Synthesis of Various Kinds of Esters by Four Microbial Lipases, *Biochim. Biophys. Acta* 575, 156–165.
14. Jensen, R.G., Dejong, F.A., and Clark, R.M. (1983) Determination of Lipase Specificity, *Lipids 18*, 239–252.
15. Macrae, A.R., and Hammond, R.C. (1985) Present and Future Applications of Lipases, *Biotechnol. Gen. Bioeng. Rev. 3*, 193–217.
16. Jencks, W.P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 289–291, Dover Publications, New York.
17. Brockerhoff, H., and Jensen, R.G. (1974) *Lipolytic Enzymes*, pp. 10–24, Academic Press, New York, San Francisco, London.
18. Penasse, L. (1974) *Les Enzymes, Cinétique et Mécanisme d'Action*, pp. 115–144, Ed Masson et Cie, Paris.

[Received March 10, 1995, and in revised form May 23, 1995; Revision accepted May 23, 1995]

Octadecatrienoic Acids as the Substrates for the Key Enzymes in Glycerolipid Biosynthesis and Fatty Acid Oxidation in Rat Liver

Takashi Ide[a,*], Masakazu Murata[a] and Michihiro Sugano[b]

[a]Laboratory of Nutrition Biochemistry, National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba 305 and [b]Laboratory of Food Science, Kyushu University School of Agriculture, Fukuoka 812, Japan

ABSTRACT: The activities of key enzymes in glycerolipid biosynthesis and fatty acid oxidation were compared using CoA esters of naturally occurring positional isomers of octadecatrienoic acids (18:3) as the substrates. The trienoic acids employed were 9,12,15-18:3 (α-18:3), 6,9,12-18:3 (γ-18:3), and 5,9,12-18:3 (pinolenic acid which is a fatty acid contained in pine seed oil, po-18:3). The activities of microsomal glycerol 3-phosphate acyltransferase obtained with various 18:3 were only slightly lower than or comparable with those obtained with palmitic (16:0), oleic (18:1), and linoleic (18:2) acids. Mitochondrial glycerol 3-phosphate acyltransferase was exclusively specific for saturated fatty acyl-CoA. The activities of microsomal diacylglycerol acyltransferase measured with various polyunsaturated fatty acyl-CoAs were significantly lower than those obtained with 16:0- and 18:1-CoAs. Among the polyunsaturated fatty acids, γ-18:3 gave the distinctly low activity. The V_{max} values of the mitochondrial carnitine palmitoyltransferase I were significantly higher with α-18:3 and po-18:3 but not γ-18:3, than with 16:0 and 18:2, while the apparent K_m values were the same irrespective of the types of acyl-CoA used except for the distinctly low value obtained with γ-18:3. The response to an inhibitor of the acyltransferase reaction, malonyl-CoA, was appreciably exaggerated with 18:2, α-18:3, and po-18:3 more than with 16:0 and 18:1. However, the response with γ-18:3 was the same as with 16:0. Thus, some of glycerolipid biosynthesis and fatty acid oxidation enzymes could discriminate not only the differences in the degree of unsaturation of fatty acids but also the positional distribution of double bond among the naturally occurring 18:3 acids.
Lipids 30, 755–762 (1995).

It has been demonstrated that the dietary fats containing octadecatrienoic acids, such as α-linolenic and γ-linolenic acids, exert various physiological activities in experimental animals and humans (1–6). It is probable that these polyunsaturated fatty acids exert their specific functions through the modification of prostanoid synthesis (1,6). However, they are not only metabolized by the enzymes in prostanoid biosynthesis but also are handled by those involved in glycerolipid biosynthesis and fatty acid oxidation. Thus, it is important to clarify the efficacies of these trienoic acids as substrates of the enzymes in the fatty acid esterification and oxidation pathways in order to understand their metabolic fates in the organisms. Available data suggested that some of the enzymes in these metabolic pathways in rat liver can discriminate the degree of unsaturation of fatty acid substrates. The activities of the mitochondrial glycerol 3-phosphate acyltransferase (EC 2.3.1.15) using oleoyl- and linoleoyl-CoAs as the substrates were distinctly lower than that obtained with palmitoyl-CoA (7–9). In contrast, the activity of microsomal glycerol 3-phosphate acyltransferase measured with palmitoyl-CoA has been demonstrated to be indistinguishable from that obtained with oleoyl-CoA (7–10) or linoleoyl-CoA (7). Rustan *et al.* (11) reported that microsomal diacylglycerol acyltransferase (EC 2.3.1.20) activity measured with eicosapentenoyl-CoA was considerably lower than that measured with oleoyl-CoA. However, various naturally occurring octadecatrienoic acids have not been tested as the substrates for these enzymes. With regard to the rate of oxidation of octadecatrienoic acids, Clouet *et al.* (12) reported that α-linolenic acid is oxidized at a rate greater than those observable with linoleic and oleic acids in isolated liver mitochondria. Gavino and Gavino (13) have reported that activity of mitochondrial carnitine palmitoyltransferase I (EC 2.3.1.21) measured with α-linolenoyl-CoA is higher than those measured with various fatty acyl-CoA substrates differing in the degree of unsaturation. As carnitine palmitoyltransferase I is an enzyme which regulates the rate of transport of fatty acids across the mitochondrial membrane (14), the substrate specificity of this enzyme accounts for the differences in the rates of oxidation in isolated mitochondria of fatty acids with differences in the degree of unsaturation (12,13). However, it is unknown whether octadecatrienoic acids, other than α-linolenic acid, with differences in the position of the double bonds, behave similarly as substrates for this enzyme. In these contexts, we currently measured the activities of glycerol 3-phosphate acyltransferase, diacylglycerol acyltransferase, and carnitine palmitoyltransferase I using three types of naturally occurring positional iso-

*To whom correspondence should be addressed at Laboratory of Nutrition Biochemistry, National Food Research Institute, 2-1-2 Kannondai, Tsukuba 305, Japan.

Copyright © 1995 by AOCS Press

mers of octadecatrienoic acids, α-linolenic, γ-linolenic and pinolenic (5,9,12-octadecatrienoic) acids, as the substrates. Pinolenic acid is rare in nature except for its presence in pine oil, and it appears not to interfere with the metabolism of linoleic acid to arachidonic acid in contrast to α-linolenic acid (15). Thus, it would be of interest to examine whether the enzymes in fatty acid esterification and oxidation distinguish these fatty acids.

MATERIALS AND METHODS

Materials. Hexadecanoic (16:0), 9-octadecenoic (18:1), and 9,12-octadecadienoic acids (18:2) were purchased from Wako Pure Chemicals (Osaka, Japan). 9,12,15-Octadecatrienoic acid (α-18:3) was obtained from Nakarai Tesk Co. (Tokyo, Japan). 6,9,12-Octadecatrienoic acid (γ-18:3) was the product of Funakoshi Chemicals Co. (Tokyo, Japan). Purities of these fatty acids as estimated by gas–liquid chromatography were greater than 98%. 5,9,12-Octadecatrienoic acid (pinolenic acid, po-18:3), which is contained in considerable amounts (14.5% of total fatty acids) in pine oil (seeds oil from *Pinus koraiensis*) (16), was purified as the methyl ester by silver-ion chromatography using commercially available solid-phase extraction column (Mega Bond Elut™ SCX; Analytical International, Cambridge, United Kingdom) as described by Christie (17). The purity of po-18:3 thus obtained was 90.0%. The major contaminants were 18:1 (1.8%), 18:2 (3.8%), and an unidentified fatty acid (2.5%). Various fatty acids were converted to the corresponding CoA esters by the method of Kawaguchi *et al.* (18) and used as the substrates for the enzymes in glycerolipid biosynthesis and fatty acid oxidation. 1,2-Dioleoylglycerophosphorylcholine and 1,2-[1-^{14}C]-dioleoylglycerophophorylcholine were purchased from Avant Polar-Lipids Laboratories (Alabaster, AL) and Amersham International (Bucks, United Kingdom), respectively. 1,2-[1-^{14}C]-Dioleoylglycerol, used as a tracer to determine the activities of rat liver microsomal diacylglycerol acyltransferase activity, was prepared by phospholipase C treatment of [^{14}C]-labeled 1,2-dioleoylglycerophosphorylcholine and purified as described previously (19). L-[methyl-^{3}H]Carnitine chloride was purchased from Amersham International. L-[U-^{14}C]Glycerol 3-phosphate (disodium salt) was the product of New England Nuclear (Boston MA). Malonyl-CoA was the product of Sigma Co. (St. Louis, MO).

Enzyme assays. Male Sprague Dawley rats, weighing 250–350 g (Charles River Japan, Kanagawa, Japan), and maintained on commercial laboratory chow, were used throughout the present study. Rats were killed by withdrawal of blood from abdominal aorta under ether anesthesia, and livers were quickly excised. A portion of the liver (approximately 3 g) was homogenized in 7 vol of 0.25 M sucrose and centrifuged at 500 × *g* for 10 min. The supernatant was recentrifuged at 9000 × *g* for 10 min to isolate mitochondria. Mitochondria were washed twice with 0.25 M sucrose containing 10 mM Tris and 1 mM EDTA (pH 7.2) and finally suspended in the same buffer or 0.25 M sucrose containing 10 mM HEPES and 1 mM EDTA (pH 7.2) for the analyses of carnitine palmitoyltransferase I (EC 2.3.1.21) and glycerol 3-phosphate acyltransferase (EC 2.3.1.15), respectively. Microsomes were isolated from 9000 × *g* supernatant by the method of Kamath and Narayan (20). The microsomes were washed once with 0.25 M sucrose to ensure the removal of cytosols and suspended in 0.25 M sucrose containing 10 mM HEPES and 1 mM EDTA (pH 7.2). Microsomal glycerol 3-phosphate acyltransferase activity was assayed using [^{14}C(U)]glycerol 3-phosphate as an acyl acceptor and various kinds of acyl-CoAs as the acyl donors according to the method of Coleman and Haynes (21) as described previously (22,23). The incubation mixture (0.2 mL) contained 4 mM $MgCl_2$, 0.4 mg bovine serum albumin (fatty acid-free), 8 mM potassium fluoride, 10–150 μM fatty acyl-CoAs, 300 μM [U-^{14}C]glycerol 3-phosphate (5000 dpm/nmol), and 55–75 μg microsomal protein in 75 mM Tris–HCl buffer (pH 7.4). Reactions were started by adding microsomes and were incubated at 37°C for 6 min. The activity of mitochondrial glycerol 3-phosphate acyltransferase activity was assayed in the presence of 1 mM 5,5′-dithio*bis* (2-nitrobenzoic acid) using 200–300 μg mitochondrial protein. It has been reported that 5,5′-dithio*bis* (2-nitrobenzoic acid) at this concentration totally inhibits microsomal, but not mitochondrial, glycerol 3-phosphate acyltransferase activity (7). The other ingredients were the same as those used for the assay of microsomal enzyme. Mitochondria were preincubated at 37°C in the mixture containing 1 mM 5,5′-dithio*bis* (2-nitrobenzoic acid) but devoid of acyl-CoA and glycerol 3-phosphate for 2 min, and the enzyme reactions were started by adding these substrates. Microsomal diacylglycerol acyltransferase activity (EC 2.3.1.20) was determined according to the method of Rustan *et al.* (11). The assay mixture (0.5 mL) contained 8 mM $MgCl_2$, 0.5 mg bovine serum albumin (fatty acid-free), 5–60 μM various acyl-CoAs, 125 μM 1,2-dioleoylglycerol dispersed in ethanol (final concentration was 10%, vol/vol), and microsomes in 175 mM Tris–HCl buffer (pH 7.8). It has been reported that 1,2-dioleoylglycerol, among the various molecular species of diacylglycerol substrates, gave the highest enzyme activity (19,24) and is commonly used as the substrate to measure the activity of this enzyme (11,21,25). The amounts of microsomes employed and the enzyme reaction time were 15–20 μg protein and 3 min, respectively. Triacylglycerols formed were extracted and separated by thin-layer chromatography using a solvent system of petroleum ether/diethyl ether/acetic acid (82:18:1, by vol) and visualized with iodine vapor. The triacylglycerol fraction was scraped off and the radioactivity was counted by a liquid scintillation counter. Carnitine palmitoyltransferase I activity was determined using freshly isolated mitochondria as an enzyme source according as the method of Bremer *et al.* (26). The assay mixture (1.0 mL) contained 82 mM sucrose, 70 mM KCl, 35 mM imidazole, 35 mM HEPES, 1 mM reduced glutathione, 2 mM KCN, 4 mg bovine serum albumin (fatty acid-free), 0.5 mM L-[methyl-^{3}H] carnitine (2220 dpm/nmol), 10–200 μM various acyl-CoAs, and mitochondria (150–250 μg protein). The pH of the

mixture was 7.0. The enzyme reaction was initiated by adding mitochondria and incubated at 30°C for 5 min. The reaction was terminated by adding 2 mL of 6% perchloric acid. After standing at least for 15 min in ice bath, the mixture was centrifuged at 2000 × *g* for 10 min. The precipitate was washed once with 2 mL of 6% perchloric acid and dissolved with 1.6 mL of water. The acylcarnitine was then extracted with 1 mL of butanol, and 0.7 mL of the butanol layer was assayed for the radioactivity by liquid scintillation counting (26). Protein was determined according to the method of Lowry *et al.* (27) using bovine serum albumin as a standard.

Statistical analysis. Data were analyzed by one-way analysis of variance, and significant differences of means were inspected by Duncan's multiple range test (28) at the level of $P < 0.05$. The statistical analyses were performed on a program developed by us using Microsoft Excel (Microsoft Corp., Redmond, WA) for the Macintosh.

RESULTS

Figure 1 and Table 1 show the activities of microsomal glycerol 3-phosphate acyltransferase using CoA esters of various fatty acids with different degree of unsaturation and positional distribution of double bonds as the substrates. The activities of the enzyme progressively increased as the concentrations of various acyl-CoAs increased up to 75–100 μM, and above these concentrations the activities decreased (Fig. 1). Table 1 summarizes the activities of the enzyme mea-

TABLE 1
Activities of Microsomal Glycerol 3-Phosphate Acyltransferase Determined with Various Acyl-CoA Substrates[a]

Acyl-CoA substrates (75 μM)	Enzyme activity[b] (nmol/min per mg protein)
16:0	1.81 ± 0.12[c]
18:1	1.61 ± 0.11[c,d]
18:2	1.59 ± 0.06[c,d]
α-18:3	1.49 ± 0.05[c,d]
γ-18:3	1.61 ± 0.07[c,d]
po-18:3	1.40 ± 0.09[d]

[a]Means ± SE for six to seven microsomal preparations obtained from different rats; po, pine seed oil.
[b]Enzyme activity represents nmoles of phosphatidic acid formed per min per mg protein.
[c,d]Values in a column not sharing a same superscript letter are significantly different at $P < 0.05$.

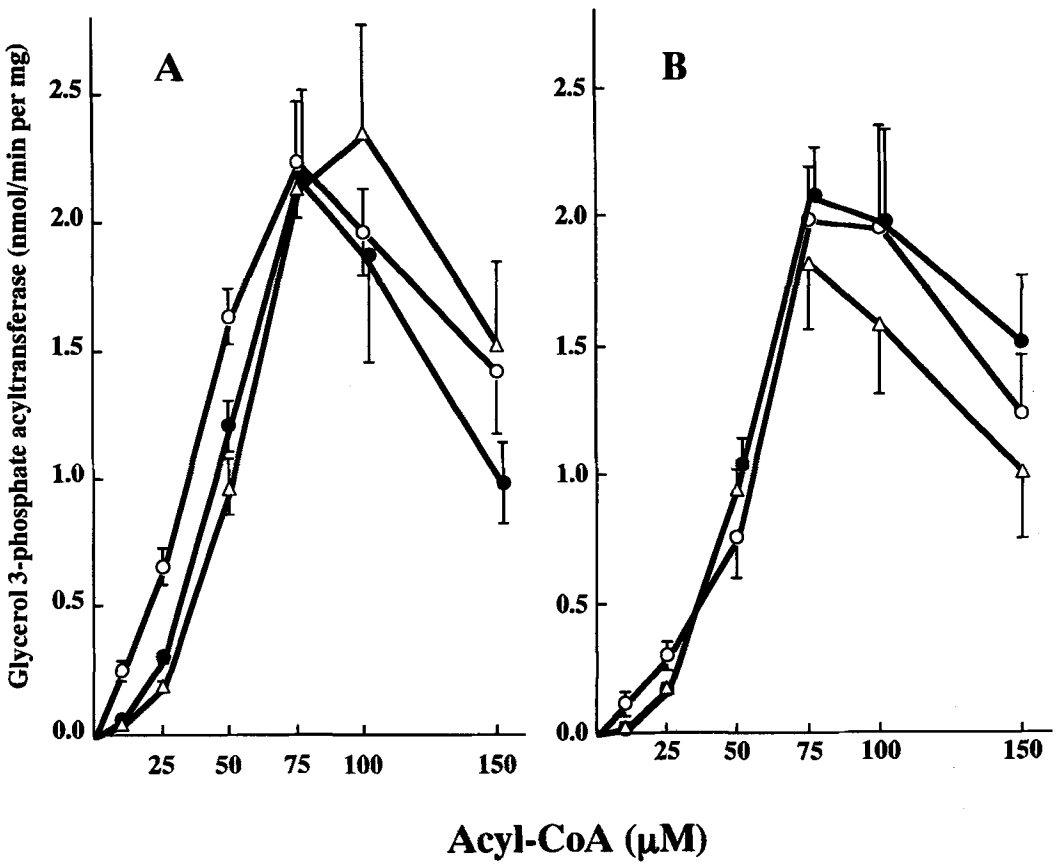


FIG. 1. Activities of microsomal glycerol 3-phosphate acyltransferase in rat liver measured with varying concentrations of various acyl-CoA substrates. Values are means ± SE of four to five microsomal preparations obtained from different rats. Enzyme activity represents nmoles of phosphatidic acid formed per min per mg protein. A: Shows the activities of the enzyme measured with 16:0- (○), 18:1- (●), and 18:2-CoA (△). B: Shows the activities of the enzyme measured with α-18:3- (○), γ-18:3- (●), and pineseed oil (po)-18:3-CoA (△).

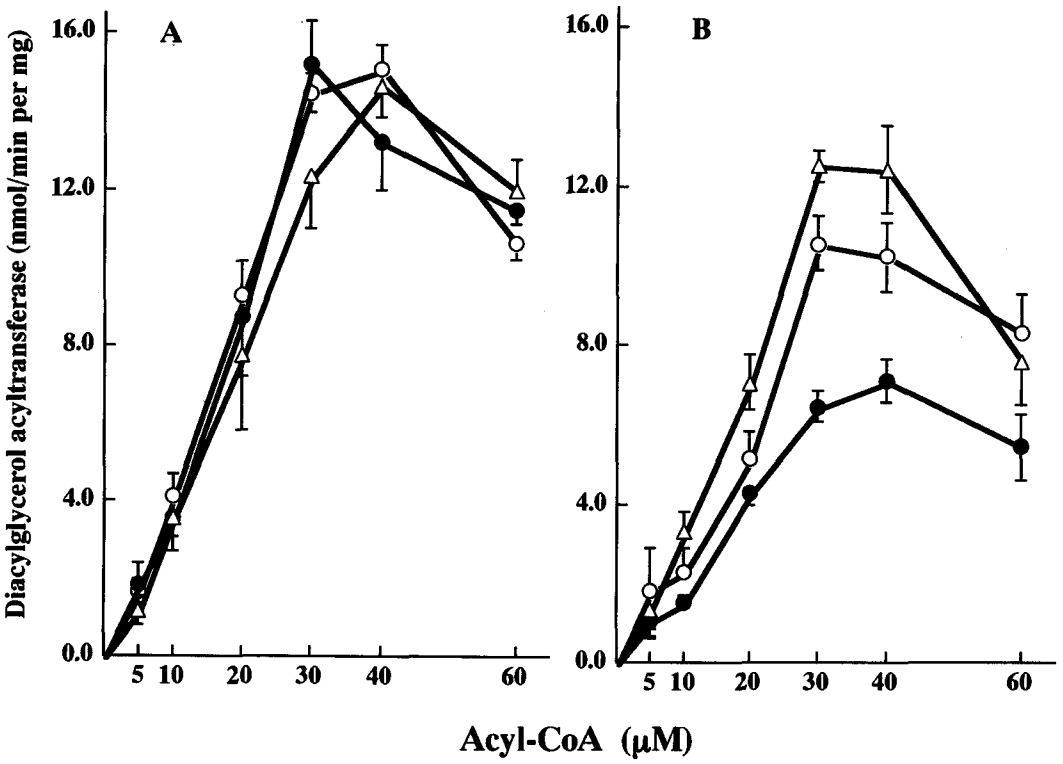


FIG. 2. Activities of microsomal diacylglycerol acyltransferase in rat liver measured with varying concentrations of various acyl-CoA substrates. Values are means ± SE of four to six microsomal preparations obtained from different rats. Enzyme activity represents nmoles of triacylglycerol formed per min per mg protein. A: Shows the activities of the enzyme measured with 16:0- (○), 18:1- (●), and 18:2-CoA (△). B: Shows the activities of the enzyme measured with α-18:3- (○), γ-18:3- (●), and po-18:3-CoA (△). See Figure 1 for abbreviation.

sured with various acyl-CoA substrates at the concentration of 75 μM using six to seven different enzyme preparations from different rats. A significant difference was detected only between the activity measured with 16:0-CoA and that measured with po-18:3-CoA. As we did not measure the activity of any mitochondrial marker enzyme in our microsomal preparations, the activities of microsomal glycerol 3-phosphate acyltransferase were assayed in the presence of 5,5-dithio*bis* (2-nitrobenzoic acid). The activities obtained were less than 0.5% of those obtained in the absence of the inhibitor, and no statistical differences were detected regardless of the types of acyl-CoA substrates employed (data not shown). This observation indicates that contamination with mitochondria of the microsomal preparation was minimal in the present study. Virtually no activity was detected in the absence of acyl-CoA substrates.

When increasing concentrations of 16:0-CoA (5–150 μM) were employed as the acyl-donor, the activities of mitochondrial glycerol 3-phosphate acyltransferase depicted a peak at the substrate concentration of 50 μM and gradually decreased above this concentration (data not shown). The average value of the activity obtained with 50 μM 16:0-CoA using six mitochondrial preparations from different rats was 230 ± 29 pmol/min per mg protein. The activities observable with various unsaturated fatty acyl-CoAs at a concentration of 50 μM were extremely low (less than 18 pmol/min per mg protein), and it was difficult to obtain reliable values using the method employed herein.

Figure 2 shows the activities of microsomal diacylglycerol acyltransferase measured with varying concentrations of acyl-CoA substrates. The concentrations of various acyl-CoAs which gave the maximal activities were 30–40 μM. Apparently, the activities obtained with γ-18:3-CoA at the various concentrations were lower than those obtained with the other acyl-CoAs. Table 2 summarizes the enzyme activities mea-

TABLE 2
Activities of Microsomal Diacylglycerol Acyltransferase Determined with Various Acyl-CoA Substrates[a]

Acyl-CoA substrates (30 μM)	Enzyme activity[b] (nmol/min per mg protein)
16:0	14.8 ± 0.5[e]
18:1	14.9 ± 0.9[e]
18:2	11.9 ± 0.9[d]
α-18:3	10.0 ± 0.5[d]
γ-18:3	6.64 ± 0.38[c]
po-18:3	11.9 ± 0.5[d]

[a]Means ± SE for six to eight microsomal preparations obtained from different rats; see Table 1 for abbreviation.
[b]Enzyme activity represents nmoles of triacylglycerol formed per min per mg protein.
[c–e]Values in a column not sharing a same superscript letter are significantly different at $P < 0.05$.

sured in the presence of various acyl-CoA substrates at the concentration of 30 μM using six to eight microsomal preparations obtained from different rats. The activities observed with polyunsaturated fatty acyl-CoAs were lower than those obtained with 16:0- and 18:1-CoAs. It was again demonstrated that γ-18:3-CoA gave the lowest activity among the various polyunsaturated fatty acyl-CoAs tested.

Figure 3 shows the activity of mitochondrial carnitine palmitoyltransferase I measured with varying concentrations of acyl-CoA substrates. Apparently, α-18:3- and po-18:3-CoAs gave enzyme activities considerably higher than those obtained with 16:0- and 18:2-CoAs at the various concentrations tested. The kinetic behavior of the enzyme using γ-18:3-CoA as a substrate was considerably different from those using other octadecatrienoyl-CoAs. The enzyme activities in the presence of a low concentration (10 μM) of γ-18:3-CoA were the same as those obtained using other octadecatrienoyl-CoAs, but the activities obtained at the concentrations higher than 50 μM were apparently lower. As the contours of changes in the enzyme activities using varying concentrations of different acyl-CoAs were found to be well-fitted with the Michaelis-Menten kinetics, V_{max} and apparent K_m values of the enzyme reaction were calculated and are summarized in Table 3. This table also presents the enzyme activities using various acyl-CoAs at the concentration of 50 μM in the absence and presence of 0.5 and 3 μM malonyl-CoA, an inhibitor of the enzyme reaction (14,29–31). The acyl-CoA concentration of 50 μM did not necessarily saturate the enzyme reaction. However, it was chosen for the comparative purpose because this concentration has been commonly employed in a number of reports for the assay of this enzyme using 16:0- or 18:1-CoA substrates (26, 29–31). V_{max}, but not K_m, values for α-18:3- and po-18:3-CoAs were significantly higher than those observable for 16:0- and 18:2-CoAs. In contrast, the V_{max} value for γ-18:3-CoA was the lowest among those observed for various acyl-CoAs tested. The apparent K_m values of the enzyme reaction were approximately the same irrespective of the types of fatty acyl-CoAs tested, except for the distinctly low value obtained with γ-18:3-CoA. The activities obtained using various acyl-CoA substrates at the concentration of 50 μM reflected the observed kinetic differences for the enzyme reaction. Thus, the activities using octadecatrienoyl-CoAs, except for γ-18:3-CoA, were significantly higher than those obtained with saturated, monounsaturated, and diunsaturated species. As expected, malonyl-CoA at the concentrations of 0.5 and 3 μM severely reduced the activities of carnitine palmitoyltransferase I irrespective of the types of acyl-CoAs used as the substrate. However, the extent of the reduction by malonyl-CoA of the enzyme reaction was apparently dependent on the type of acyl-CoA substrate. Thus, the sensitivities to this inhibitor obtained with 18:2-, α-18:3-, and po-18:3-CoAs were appreciably exaggerated compared to those obtained with 16:0- and 18:1-CoAs. The response obtained with γ-18:3-CoA was unique among

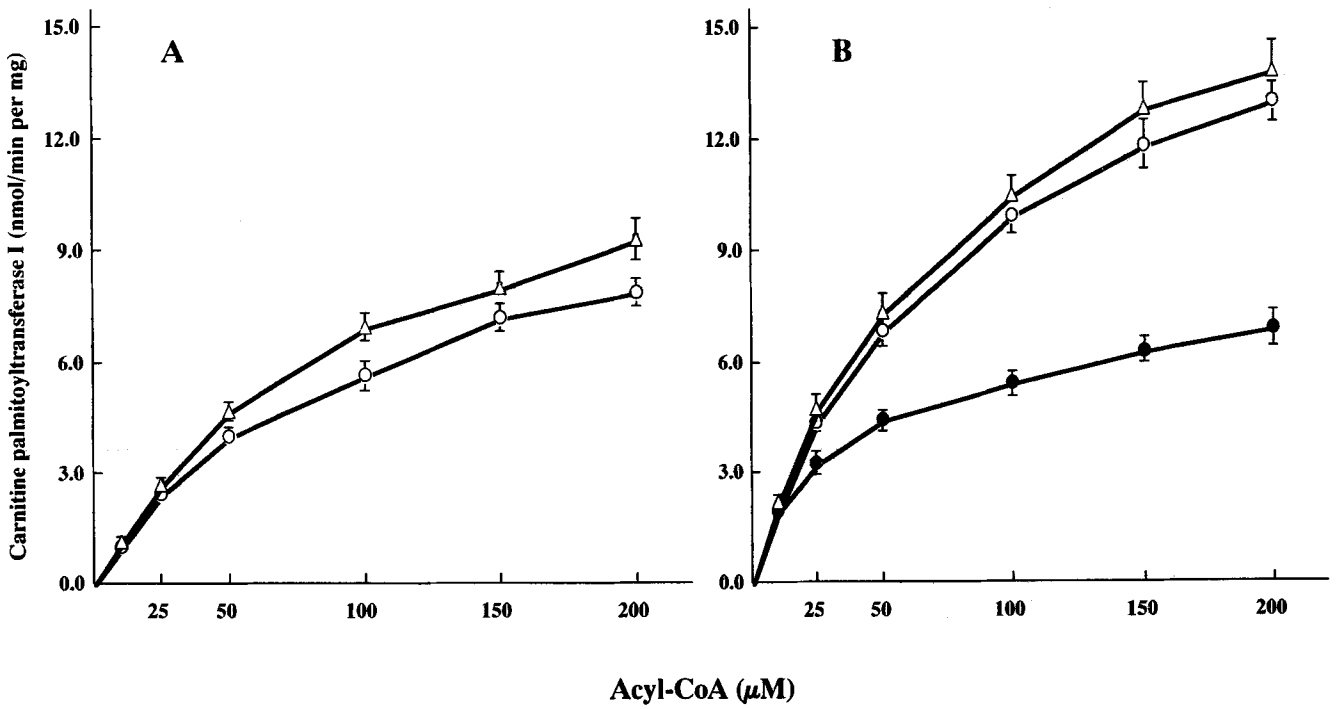


FIG. 3. Activities of mitochondrial carnitine palmitoyltransferase I in rat liver measured with varying concentrations of various acyl-CoA substrates. Values are means ± SE of six to eight mitochondrial preparations obtained from different rats. Enzyme activity represents nmoles of acylcarnitine formed per min per mg protein. A: Shows the activities of the enzyme measured with 16:0- (○), and 18:2-CoA (△). B: Shows the activities of the enzyme measured with α-18:3- (○), γ-18:3- (●), and po-18:3-CoA (△). See Figure 1 for abbreviations.

TABLE 3
Activities of Carnitine Palmitoyltransferase I in Rat Liver Mitochondria Measured with Various Acyl-CoA Substrates[a]

Acyl-CoA substrates	Enzyme activities in the presence of malonyl-CoA at the concentrations (μM) of[b] (nmol/min per mg protein)					V_{max}[b] (nmol/min per mg protein)	Apparent K_m (μM)
	0	0.5		3.0			
16:0	4.01 ± 0.15^{d}	3.07 ± 0.26^{d,e}	(75.8 ± 3.4^{g})	1.36 ± 0.16^{c,d}	(33.0 ± 3.0^{d})	11.9 ± 0.6^{d}	103 ± 7^{d}
18:1	3.02 ± 0.09^{c}	1.94 ± 0.08^{c}	(63.0 ± 2.7^{d,e,f})	0.87 ± 0.04^{c}	(28.4 ± 1.7^{d})	ND	ND
18:2	4.80 ± 0.19^{e}	2.57 ± 0.33^{c,d}	(52.7 ± 4.9^{c,d})	0.96 ± 0.15^{c}	(19.5 ± 2.4^{c})	13.5 ± 0.8^{d}	99.3 ± 5.0^{d}
α-18:3	6.86 ± 0.25^{f}	3.35 ± 0.44^{d,e}	(49.2 ± 4.9^{c})	1.07 ± 0.13^{c}	(15.8 ± 1.5^{c})	19.3 ± 0.6^{e}	86.0 ± 10.0^{d}
γ-18:3	4.38 ± 0.16^{d,e}	3.29 ± 0.28^{d,e}	(73.9 ± 3.8^{f,g})	1.67 ± 0.24^{d}	(36.8 ± 4.3^{d})	7.99 ± 0.50^{c}	40.5 ± 6.9^{c}
po-18:3	7.15 ± 0.30^{f}	3.96 ± 0.39^{e}	(54.8 ± 4.2^{c,e})	1.26 ± 0.16^{c,d}	(17.4 ± 2.0^{c})	19.8 ± 0.9^{e}	83.7 ± 8.3^{d}

[a]Values are means ± SE of six to ten mitochondrial preparations obtained from different rats. Percentages of the value obtained in the absence of malonyl-CoA are in parentheses. See Table 1 for abbreviation.
[b]Enzyme activities and V_{max} values represent nmoles of acylcarnitine formed per min per mg protein.
[c–g]Values in a column not sharing a common superscript letter are significantly different at $P < 0.05$. ND, not determined.

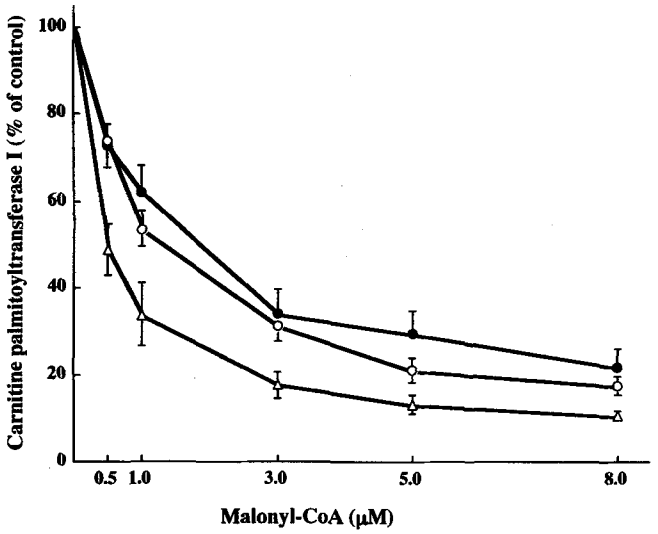


FIG. 4. Activities of mitochondrial carnitine palmitoyltransferase I in rat liver obtained in the presence of varying concentrations of malonyl-CoA. Values are means ± SE of five to six mitochondrial preparations obtained from different rats. Percentages of the value obtained in the absence of malonyl-CoA using 50 μM of 16:0- (○), 18:2- (△), and γ-18:3-CoA (●) as the substrates are presented.

the various acyl-CoAs of polyunsaturated species, and the sensitivity to malonyl-CoA was the same as those obtained with 16:0- and 18:1-CoAs. The data presented in Figure 4 confirmed this observation. Thus, the extent of the reduction in the presence of varying concentrations of malonyl-CoA using 16:0- and γ-18:3-CoAs in relation to 18:2-CoA were appreciably attenuated.

DISCUSSION

This study demonstrated that some of the key enzymes in fatty acid esterification and oxidation pathways of rat liver can discriminate not only the various octadecatrienoic acids from other fatty acids differing in the degree of unsaturation, but also the individual trienoic acids examined.

Substrate specificity of glycerol 3-phosphate acyltransferase. Previous studies have demonstrated that the activities of microsomal glycerol 3-phosphate acyltransferase obtained with 16:0-, 18:1-, and 18:2-CoAs were indistinguishable from each other (7–10). Although the activities of this enzyme tended to decrease with an increase in the degree of unsaturation of acyl-CoA substrates employed in the present study, this enzyme apparently showed a broad specificity toward the various acyl-CoAs with differences in the degree of unsaturation and the position of the double bonds. Marked selectivity of mitochondrial glycerol 3-phosphate acyltransferase toward saturated fatty acyl-CoA compared to 18:1- and 18:2-CoAs (7–9) was confirmed in the present study. In addition, we expanded the observation that various octadecatrienoyl-CoAs were not effective as the substrates for mitochondrial glycerol 3-phosphate acyltransferase. Thus, along with the observations made by others (7–10), it is suggested that the microsomal, but not mitochondrial, glycerol 3-phosphate acyltransferase is rather incapable of discriminating the differences in the degree of unsaturation and positional distribution of double bonds in acyl moieties of the substrate.

Substrate specificity of diacylglycerol acyltransferase. Coleman and Bell (24) showed that the activity of microsomal diacylglycerol acyltransferase was dependent on the chain length of saturated fatty acyl-CoAs. However, the efficacies of acyl-CoA substrates differing in the degree of unsaturation as the substrates of this enzyme have not been the subjects for the extensive studies. Our present observation using various unsaturated fatty acyl-CoAs with 18 carbon chain length clearly demonstrated that this enzyme can discriminate the degree of unsaturation of fatty acyl-CoAs. As the activity measured with γ-18:3-CoA was distinctly lower than that observed with α-18:3- and po-18:3-CoAs, this enzyme apparently discriminated the difference in the positional distribution of double bonds as well.

Substrate specificity of carnitine palmitoyltransferase I. Consistent with a previous finding (13), the activity of carnitine palmitoyltransferase I measured with α-18:3 was higher than those obtained with 16:0, 18:1, and 18:2. Also, the activity measured with po-18:3, but not γ-18:3, was comparable

with that obtained with α-18:3. Thus, this enzyme apparently discriminated not only the degree of unsaturation but also the positional distribution of double bonds of fatty acyl-CoA substrates.

Malonyl-CoA-mediated inhibition of carnitine palmitoyltransferase I represents a mechanism by which mitochondrial oxidation of long-chain fatty acids is regulated (14). The sensitivity of the enzyme to this inhibitor has been demonstrated to be influenced by the nutritional and physiological state of animals (14). In addition, a variety of *in vitro* conditions can modulate the sensitivity of this enzyme to malonyl-CoA (29–31). The present study for the first time presents evidence that the type of acyl-CoA used as the substrate is a factor to modify *in vitro* the response of the enzyme to malonyl-CoA. Our data also suggested that the component(s) which regulate the sensitivity to malonyl-CoA discriminate not only the degree of unsaturation but also the positional distribution of double bonds of acyl-CoA substrates.

The present observation indicated that at least diacylglycerol acyltransferase and carnitine palmitoyltransferase I among the various enzymes in glycerolipid biosynthesis and fatty acid oxidation can discriminate the various positional isomers of octadecatrienoic acids from saturated, monounsaturated, and diunsaturated fatty acids. Moreover, the responses of these enzymes to these octadecatrienoic acid substrates were modified by the differences in the positional distribution of double bonds in the acyl-moieties. Although the physiological significance of the present observation remains to be clarified, several studies indicated that at least the rates of oxidation *in vivo* of octadecatrienoic acids are different from other fatty acids differing in the degree of unsaturation. Bandyopadhyay *et al.* (32) compared the fate of [1-^{14}C]-labeled 18:2 and α-18:3 *in vivo* in the liver of cat fish. They demonstrated that saturated fatty acids were preferentially labeled in the liver when the octadecatrienoic acid was a tracer, indicating that the rate of β-oxidation to [^{14}C]acetyl-CoA was higher than with 18:2. Leyton *et al.* (33) reported that the rate of the oxidation *in vivo* of orally administered [1-^{14}C]-labeled various unsaturated fatty acids, as estimated by radioactivities recovered in carbon dioxide during a 24-h period, was in the order of 18:1 = α-18:3 > 18:2 > γ-18:3 > dihomo-γ-18:3 = 20:4 in the rat. The result of this study may be compatible with our observation that mitochondrial carnitine palmitoyltransferase I can discriminate not only the degree of unsaturation but also the positional distribution of double bonds in the acyl moieties. However, a recent report (34) indicated that [1-^{14}C]-labeled 18:1, 18:2, and α-18:3 are oxidized at similar rates in the rat given a purified 15% fat diet containing equal amounts of 18:1, 18:2, and α-18:3 (23.2, 22.7, and 23.0%, respectively). In the present study, we demonstrated that the response to malonyl-CoA of carnitine palmitoyltransferase I is dependent on the types of fatty acids used as the substrates. Thus, it is supposed that the relative rates of the oxidation of fatty acids are influenced by the nutritional and physiological states which modify the malonyl-CoA concentration in the tissues. Thus, differences in the tissue malonyl-CoA concentrations may account for the discrepancy in the rates of oxidation *in vivo* of polyunsaturated fatty acids between rats fed commercial (33) and purified diets (34).

Fatty acyl-CoA is an amphiphilic molecule and forms monomer solutions only at a low concentration. At the concentrations above the critical micelle concentration, it forms micelles. Available data suggested that critical micelle concentrations of fatty acyl-CoAs decrease as the carbon number increases. On the other hand, the presence of a double bond increases the critical micelle concentration (35–37). However, the concentrations of various acyl-CoAs as monomers or in micelles in our complex system for the assays of respective enzymes containing enzyme protein, albumin, and various ingredients remain to be defined. Also, the information regarding the differences in the position of double bonds which affect the critical micelle concentrations of polyunsaturated fatty acyl-CoAs has been lacking. The possibility that the differences in the specificities of the enzymes for acyl-CoA substrates observed in the present study represent the features of some irregularities in the physical behaviors of various acyl-CoA substrates (35,37), rather than those of enzymes, may not be disregarded. Further studies may be required to clarify this point.

REFERENCES

1. Simopoulos, A.P. (1989) Summary of the NATO Advanced Research Workshop on Dietary ω-3 and ω-6 Fatty Acids: Biological Effects and Nutritional Essentiality, *J. Nutr. 119*, 521–528.
2. Sugano, M., Ide, T., Ishida, T., and Yoshida, K. (1986) Hypocholesterolemic Effect of Gamma-Linolenic Acid as Evening Primrose Oil in Rats, *Ann. Nutr. Metab. 30*, 289–299.
3. Hoffman, P., Block, H.-U., Beitz, J., Taube, Ch., Forster, W., Wortha, P., Singer, P., Naumann, E., and Heine, H. (1986) Comparative Study of the Blood Pressure Effects of Four Different Vegetable Fats on Young, Spontaneously Hypertensive Rats, *Lipids 21*, 733–737.
4. Yamamoto, N., Saitoh, M., Moriuchi, A., Nomura, M., and Okuyama, H. (1987) Effect of Dietary α-Linolenate/Linoleate Balance on Brain Lipid Compositions and Learning Ability of Rats, *J. Lipid Res. 28*, 144–151.
5. Bourre, J.-M., Francois, M., Youyou, A., Dumont, O., Picotti, M., Pascal, G., and Durand, G. (1989) The Effects of Dietary α-Linolenic Acid on the Composition of Nerve Membranes, Enzymatic Activity, Amplitude of Electrophysiological Parameters, Resistance to Poisons and Performance of Learning Tasks in Rats, *J. Nutr. 119*, 1880–1882.
6. Craig-Schmidt, M.C., Faircloth, S.A., and Weete, J.D. (1987) Modulation of Avian Lung Eicosanoids by Dietary Omega-3 Fatty Acids, *J. Nutr.117*, 1197–1206.
7. Yamada, T., and Okuyama, H. (1978) Selectivity of Diacylglycerophosphate Synthesis in Subcellular Fractions of Rat Liver, *Arch. Biochem. Biophys. 190*, 409–420.
8. Stern, W., and Pullman, M.E. (1978) Acyl-CoA: *sn*-Glycerol-3-Phosphate Acyltransferase and the Positional Distribution of Fatty Acids in Phospholipids of Cultured Cells, *J. Biol. Chem. 253*, 8047–8055.
9. Haldar, D., Tso, W.-W., and Pullman, M.E. (1979) The Acylation of *sn*-Glycerol 3-Phosphate in Mammalian Organs Ehrlich Ascites Tumor Cells, *J. Biol. Chem. 254*, 4502–4509.

10. Tamai, Y., and Lands, W.E.M. (1974) Positional Specificity of *sn*-Glycerol 3-Phosphate Acylation During Phosphatidate Formation by Rat Liver Microsomes, *J. Biochem. 76*, 847–860.
11. Rustan, A.C., Nossen, J.Ø., and Christiansen, E.N. (1988) Eicosapentaenoic Acid Reduces Hepatic Synthesis and Secretion of Triacylglycerol by Decreasing the Activity of Acyl-Coenzyme A: 1,2-Diacylglyceol Acyltransferase, *J. Lipid Res. 29*, 1417–1426.
12. Clouet, P., Niot, I., and Bézard, J. (1989) Pathway of α-Linolenic Acid Through the Mitochondrial Outer Membrane in the Rat Liver and Influence on the Rate of Oxidation, *Biochem. J. 263*, 867–873.
13. Gavino, R.G., and Gavino, V.C. (1991) Rat Liver Outer Mitochondrial Carnitine Palmitoyltransferase Activity Towards Long-Chain Polyunsaturated Fatty Acids and Their CoA Esters, *Lipids 26*, 266–270.
14. Shulz, H. (1991) Beta Oxidation of Fatty Acids, *Biochim. Biophys. Acta 1081*, 109–120.
15. Sugano, M., Ikeda, I., Wakamatsu, K., and Oka, T. (1994) Influence of Korean Pine (*Pinus koraiensis*)-Seed Oil Containing *cis*-5,*cis*-9,*cis*-12-Octadecatrienoic Acid on Polyunsaturated Fatty Acid Metabolism, Eicosanoid Production and Blood Pressure of Rats, *Br. J. Nutr. 72*, 775–783.
16. Takagi, T., and Itabashi, Y. (1982) *cis*-Olefinic Unusual Fatty Acids in Seed Lipids of Gymnospermae and Their Distribution in Triacylglycerols, *Lipids 17*, 716–723.
17. Christie, W.W. (1989) Silver Ion Chromatography Using Solid-Phase Extraction Columns Packed with a Bonded-Sulfonic Acid Phase, *J. Lipid Res. 30*, 1471–1473.
18. Kawaguchi, A., Yoshimura, T., and Okuda, S. (1981) A New Method for the Preparation of Acyl-CoA Substrates, *J. Biochem. 71*, 576–581.
19. Ide, T., and Murata, M. (1993) The Acyl-Acceptor Specificity of Microsomal Diacylglycerol Acyltransferase as a Possible Determinant in Regulating Hepatic Triacylglycerol Synthesis in Rats Fed a Polyunsaturated Fat Diet, *J. Nutr. Biochem. 4*, 229–235.
20. Kamath, S.A., and Narayan, K.A. (1972) Interaction of Ca^{2+} with Endoplasmic Reticulum of Rat Liver. A Standarized Procedure for the Isolation of Rat Liver Microsomes, *Anal. Biochem. 48*, 53–61.
21. Coleman, R.A., and Haynes, E.B. (1983) Selective Changes in Microsomal Enzymes of Triacylglycerol Synthesis in Fetal and Post-Natal Rat Liver. Induction of Microsomal Glycerol 3-Phosphate and Dihydroxyacetone Phosphate Acyltransferase Activities, *J. Biol. Chem. 258*, 450–456.
22. Ide, T., Murata, M., and Sunada, Y. (1992) Soybean Protein-Dependent Changes in Triacylglycerol Synthesis and Concentration of Diacylglycerol in the Liver Microsomes of Fasted-Refed Rats, *Ann. Nutr. Metab. 36*, 87–96.
23. Ide, T., Hirabayashi, S., Kano, S., and Sugano, M. (1992) Soybean Phospholipid Dependent Reductions in Triacylglycerol Concentration and Synthesis in the Liver of Fasted-Refed Rats, *Biochim. Biophys. Acta 1124*, 163–170.
24. Coleman, R., and Bell, R.M. (1976) Triacylglycerol Synthesis in Isolated Fat Cells. Studies on the Microsomal Diacylglycerol Acyltransferase Activity Using Ethanol-Dispersed Diacylglycerol, *J. Biol. Chem. 251*, 4537–4543.
25. Iritani, N., Suga, A., Fukuda, H., Katsurada A., and Tanaka, T. (1988) Effect of Dietary Casein and Soybean Protein on Triglyceride Turnover in Rat Liver, *J. Nutr. Sci. Vitaminol. 34*, 309–315.
26. Bremer, J., Woldegiorgis, G., Schhalinske, K., and Shargo, E. (1985) Carnitine Palmitoyltransferase. Activation by Palmitoyl–CoA and Inactivation by Malonyl-CoA, *Biochim. Biophys. Acta 833*, 9–16.
27. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurements with Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
28. Duncan, D.B. (1957) Multiple Range Test for Correlated and Heteroscedastic Means, *Biometrics 13*, 164–176.
29. Stephens, T.W., Cook, G.A., and Harris, R.A. (1983) Effect of pH on Malonyl–CoA Inhibition of Carnitine Palmitoyltransferase I, *Biochem. J. 212*, 521–524.
30. Zammit, V.A., Corstorphine, C.G., and Gray, S.R. (1984) Changes in the Ability of Malonyl-CoA to Inhibit Carnitine Palmitoyltransferase I Activity and to Bind to Rat Liver Mitochondria During Incubation *in vitro*, *Biochem. J. 222*, 335–342.
31. Cook, G.A. (1984) Involvement of Hysteretic Effects in the Inhibition of Carnitine Palmitoyltransferase by Malonyl-CoA, *Biochem. J. 224*, 1015–1018.
32. Bandyopadhyay, G.K., Dutta, J., and Ghosh, S. (1982) Preferential Oxidation of Linolenic Acid Compared to Linoleic Acid in the Liver of Catfish (*Heteropneustes fossilis* and *Clarias batrachus*), *Lipids 17*, 733–740.
33. Leyton, J., Druty, P.J., and Crawford, M.A. (1987) Differential Oxidation of Saturated and Unsaturated Fatty Acids *in vivo* in the Rat, *Br. J. Nutr. 57*, 383–393.
34. Jones, P.J.H. (1994) Dietary α-Linolenic and Oleic Acids Are Oxidized at Similar Rates in Rats Fed a Diet Containing These Acids in Equal Proportions, *Lipids 29*, 491–495.
35. Barden, R.E., and Cleland, W.W. (1969) 1-Acylglycerol 3-Phosphate Acyltransferase from Rat Liver, *J. Biol. Chem. 244*, 3677–3684.
36. Powell, G.L., Grothusen, J.R., Zimmerman, J.K., Evans, C.A., and Fish, W.W. (1981) A Re-Examination of Some Properties of Fatty Acyl-CoA Micelles, *J. Biol. Chem. 256*, 12740–12747.
37. Smith, R.H., and Powell, G.L. (1986) The Critical Micelle Concentration of Some Physiologically Important Fatty Acyl-Coenzyme A's as a Function of Chain Length, *Arch. Biochem. Biophys. 244*, 357–360.

[Received November 23, 1994; in revised form April 25, 1995; Revision accepted May 18, 1995]

Inhibition of Neutral Cholesteryl Ester Hydrolase by the Glycolytic Enzyme Enolase. Is This a Secondary Function of Enolase?

John H. Shand and David W. West*

Hannah Research Institute, Ayr, KA6 5HL, Scotland, United Kingdom

ABSTRACT: There is an accumulation of the glycolytic enzyme enolase and of cholesteryl esters in macrophages that have been converted into "foam" cells. In this study, we questioned whether enolase could be involved in this accumulation of cholesteryl esters by inhibiting the activity of neutral cholesteryl ester hydrolases. Enolase from both yeast and rabbit muscle were incubated with three different cholesteryl ester hydrolases and were shown to inhibit the hydrolysis of cholesteryl esters. Inhibition was dependent on the concentration of enolase and appeared to occur through binding of the enolase to the cholesteryl ester. Nevertheless, the yeast and rabbit muscle enolases differed in their efficiency of inhibition and in their mechanism of action. Purification of commercial enolase preparations by gel-filtration yielded single proteins with the same inhibitory activities as the originals, indicating that the inhibition was not due to the presence of an impurity. Partially purified αα- and γγ-isoforms of the enzyme from rat brain also appear to have inhibitory effects on cholesteryl ester hydrolysis. Negative control of the hydrolytic phase of the cholesterol/cholesteryl ester cycle may be a secondary function of enolases which correlates with the accumulation of cholesteryl esters in a number of neuro-degenerative and demyelinating diseases.
Lipids 30, 763–770 (1995).

Cholesterol is a molecule critical to the structural and functional integrity of all mammalian cells, and the levels of the free sterol are tightly regulated. Excess cholesterol is esterified with fatty acyl–CoA derivatives in a reaction catalyzed by acyl CoA–cholesterol acyltransferase (1–3), whereas free sterol is released from these cholesteryl ester stores by hydrolysis catalyzed by a neutral cholesterol ester hydrolase (CEH). Thus, a continuous cycle of esterification and hydrolysis exists within the cell (4,5).

Many disease states are characterized by accumulations of cholesteryl esters, the classic case being atherosclerosis, in which macrophages accumulate large amounts of cholesteryl esters to form the "foam" cells which are found in atherosclerotic lesions (5–8). This accumulation has been postulated to arise from an increased intake of cholesterol (5), but, as there is a continual cycle of esterification and hydrolysis, a number of other factors may also affect this process. These could include alteration in the activity of the CEH, and we have recently demonstrated that a number of cells, including hepatocytes (9) and macrophages (10), contain a cytosolic protein inhibitor of this enzyme. The inhibitor activity could be altered by physiological, hormonal, and nutritional manipulation (9,11). In particular, we demonstrated that under conditions that favored the conversion of macrophages into "foam" cells, the activity of this inhibitor was increased (10).

More recently, Tabas and co-workers have shown that when macrophages were converted into "foam" cells by incubation with acetylated low-density lipoproteins (12), the expression of a number of cellular proteins was altered. Two proteins were observed to be increased in amount and one of these, which showed a 458% increase, was shown to have structural homology with the α-isoform of the glycolytic enzyme enolase and to be immunoprecipitated by antiserum specific for α-enolase. Although this form of α-enolase did not appear to have enolase activity and no function was ascribed to it, the similarity between our results with macrophages and those reported by Bottalico *et al.* (12) raised the possibility that the increased inhibitory activity observed in our experiments may have been caused by the increased enolase protein. This hypothesis was given support by the fact that, during attempts to purify the cytosolic inhibitor from rat liver, it became apparent that its behavior, on anion exchange chromatography, showed a strong resemblance to that exhibited by the isoforms of enolase (13,14).

We have therefore investigated the effect of both mammalian and yeast enolases on cholesteryl ester hydrolysis by two different mammalian and one yeast CEH. Our results indicate that the enolase molecule has the ability to bind cholesteryl esters and, in doing so, to inhibit their hydrolysis by the CEH. Binding occurred even in the presence of an excess of bovine serum albumin (BSA) or cytosolic proteins, suggesting that this inhibition could also occur *in vivo*.

*To whom correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; CEH, cholesteryl ester hydrolase; FPLC, fast-performance liquid chromatography; HSL, hormone-sensitive lipase; PAGE, polyacrylamide gel electrophoresis..

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Baker's yeast enolase was purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom) and the *Candida cylindracea* CEH (EC 3.1.1.13) from BCL (Lewes, Sussex, United Kingdom). Rabbit muscle enolase (EC 4.2.1.11) was purchased from three sources: ICN (High Wycombe, Bucks, United Kingdom), as well as from Sigma and BCL. Cholesteryl [1-^{14}C]oleate was purchased from Amersham (Little Chalfont, Bucks, United Kingdom), and cholesteryl [9,10-^{3}H]-oleate was prepared as previously described (9). Other biochemicals were obtained from Sigma or BCL. Purified bovine adipose tissue hormone-sensitive lipase (HSL) was kindly supplied by Professor S.J. Yeaman (University of Newcastle, Newcastle-Upon-Tyne, United Kingdom).

Tissue fractionation. Microsomes were prepared from the mid-lactating mammary glands of female Wistar rats and from the livers of male Wistar rats, as described previously (9). An acetone–diethyl ether powder was also prepared from the rat liver cytosolic fraction (9).

Preparation of rat brain enolases. Rat brains were homogenized in 15 mM Tris-acetate buffer (pH 6.5) containing 5 mM $MgSO_4$ and 0.1 mM EDTA. The homogenate was centrifuged and treated with Macro-prep CM (Biorad, Hemel Hempstead, Herts, United Kingdom) and ammonium sulfate exactly as described by Suzuki *et al.* (15). The 40–80% ammonium sulfate fraction was passed through Sephadex G-15 (Pharmacia, Uppsala, Sweden) and applied to a column of Macro-prep Q (Biorad) equilibrated in homogenization buffer. The nonretained material was collected (αα-enolase), and further enolase fractions were obtained by washing the column with homogenization buffer containing first 100 mM NaCl (αγ-enolase) and then 220 mM NaCl (γγ-enolase).

Purification of commercial enolase preparations. Rabbit muscle enolase, supplied as a suspension in ammonium sulfate, was centrifuged (10,000 × *g*, 10 min) and the pellet dissolved in 50 mM 2-(*N*-morpholino)-ethanesulfonic acid, 50 mM TRIS buffer, pH 7.2, containing 1 mM EDTA. Baker's yeast enolase was dissolved directly in the same buffer. Both enolase preparations were routinely passed through a small column (2 mL) of Sephadex-G15 (Pharmacia), equilibrated with the same buffer before use. In addition, for some experiments, both enolases were further purified by fast-performance liquid chromatography (FPLC) on a Superose-12 gel-filtration column (Pharmacia) equilibrated in the same buffer. Purity was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) using both the discontinuous buffer systems of Laemmli (16) and of Amory *et al.* (17), as well as the continuous system of Fairbanks *et al.* (18).

Assay of enzyme activities. CEH activity was assayed (total volume 0.2 mL) using either mammary gland microsomal membranes (100 μg), liver microsomal membranes (50 μg), *C. cylindracea* CEH (25 ng), or HSL (160 ng) in an assay buffer consisting of 50 mM MES, 50 mM TRIS/HCl buffer, pH 7.2, and 0.5 mM EDTA. Fatty acid-free BSA (200 μg) and enolase (0.004–3.6 nmol) were added to the assay mixture, and the reaction was initiated by the injection of an ethanolic solution of cholesteryl [9,10(n)-^{3}H]oleate (20 nmol, 200,000 dpm, 10 μL). Where indicated in the text, the reaction was also initiated by the addition of the mammary gland microsomal membranes, suspended in a solution of BSA, to the incubation tubes containing assay buffer, enolase, and substrate. After 20 min at 37°C, the reaction was terminated and the extent of hydrolysis of the substrate determined as previously described (9). Assays were performed in triplicate, and appropriate controls, without microsomes, were incubated concurrently to correct for background. Enolase activity was measured at 30°C using the coupled assay as described previously (15).

Assay of cholesteryl oleate binding. Enolase (0–1.5 nmol) was preincubated for 10 min at 37°C in the assay buffer, with and without BSA (200 μg), before binding was initiated, by the addition of an ethanolic solution of either cholesteryl [9,10-^{3}H]oleate (20 nmol, 290,000 dpm) or cholesteryl [1-^{14}C]oleate (20 nmol, 103,000 dpm) to give a final volume of 0.2 mL. After a further 10 min at 37°C, the reaction was either terminated immediately, by cooling the mixture in ice for 5 min or, where indicated, BSA (200 μg) was added and incubation continued for a further 10 min prior to termination. The reaction mixture was then centrifuged (15,000 × *g*, 25 min), and duplicate aliquots of the supernatant counted to determine the amount of cholesteryl oleate remaining in solution. Assays were performed in triplicate.

Protein determination. Protein was determined by the method of Bradford (19) using BSA as a standard.

RESULTS

The CEH activity present in the mammary gland microsomes was inhibited when assayed in the presence of rabbit muscle enolase (predominantly the ßß-isoform of the enzyme) (Fig. 1A). Consistent results were obtained only when low molecular weight material was removed from the enolase preparation by passage of the protein through Sephadex-G15. Inhibition was dependent on the concentration of rabbit muscle enolase, but total inhibition of hydrolytic activity was not achieved. Linear regression analysis of the first portion of the graph indicated that 50% inhibition of the CEH activity required 0.56 nmol of muscle enolase (assuming M_r of 45,000). A purified sample of HSL, which has immunological identity with the mammary gland CEH (20), was similarly inhibited (0.76 nmol of muscle enolase gave 50% inhibition).

The CEH activity in liver microsomes (50 μg), which has been shown to be due to a different enzyme from HSL (21,22), was more sensitive to inhibition by the muscle enolase, in as much as 50% inhibition required only 0.33 nmol of the enolase (Fig. 1A). The CEH from *C. cylindracea* was relatively insensitive, 50% inhibition required 5.09 nmol of the enolase (Fig. 1A). Whereas the assay was normally initiated by the addition of substrate to a mixture of enolase, BSA and the mammary microsomal CEH, when rabbit muscle enolase was preincubated with the cholesteryl ester for 10 min, prior to the addition of BSA and the mammary gland microsomal

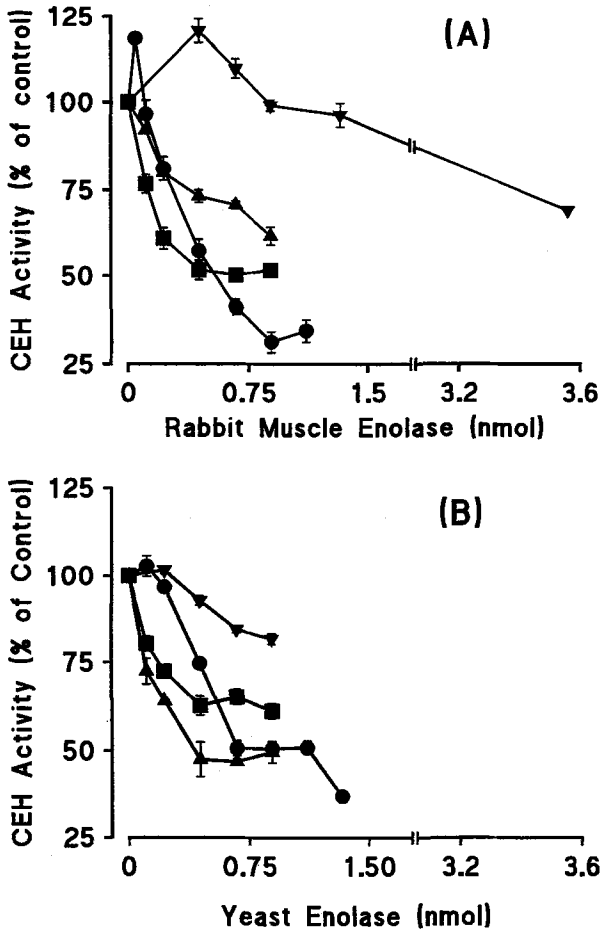


FIG. 1. Variation of the inhibition of neutral cholesteryl ester hydrolases (CEH) with the concentration of (A) rabbit muscle enolase and (B) yeast enolase. Mammary microsomes (100 μg, ▲), liver microsomes (50 μg, ■), hormone-sensitive lipase (HSL) (●), and esterase from *Candida cylindracea* (▼) were assayed for CEH activity in the presence of varying amounts of either rabbit muscle enolase or yeast enolase. Reaction was initiated by the addition of an ethanolic solution of cholesteryl [^{3}H]oleate as indicated in the Materials and Methods section. The control (100%) values for HSL and *C. cylindracea* were 0.384 and 2.95 μmol/min/mg, respectively, and for the microsomes from mammary gland and liver 459.2 ± 7.9 and 76.5 ± 5.6 pmol cholesteryl oleate hydrolyzed per min/mg of protein, respectively. The results are typical of a number of experiments and are the means ± SEM for triplicate measurements in each instance.

CEH, 50% inhibition of hydrolytic activity was achieved with only 10.9 pmol of the enolase. (Fig. 2).

Inhibition by yeast enolase showed a different pattern of sensitivity (Fig. 1B). The mammary gland microsomal CEH required 0.21 nmol of the yeast enolase to produce 50% inhibition, whereas the liver microsomal enzyme required 0.39 nmol. The *C. cylindracea* CEH required 1.51 nmol for 50% inhibition, and HSL required 0.59 nmol for the same effect.

The activity of the mammary microsomal CEH against triolein was also inhibited by yeast and rabbit muscle enolase (40.5 ± 3.3 and 46.4 ± 1.8% inhibition, respectively, with 0.81 nmol of enolase), whereas the activity of the purified HSL

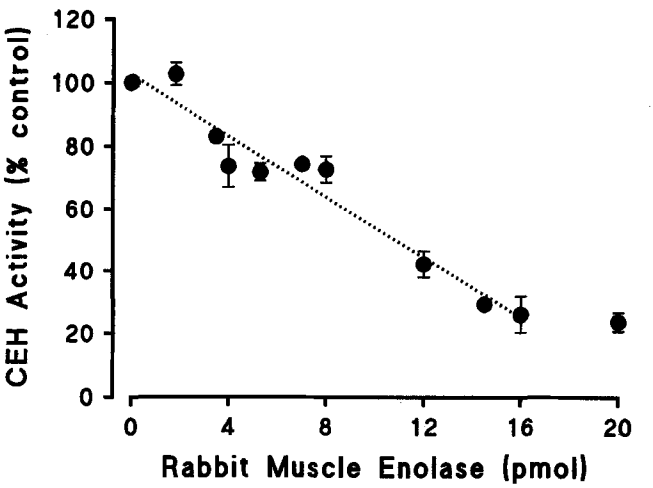


FIG. 2. Variation of the inhibition of cholesteryl ester hydrolysis by rabbit muscle enolase: effect of preincubating enolase and the cholesteryl ester. Rabbit muscle enolase (0–20 pmol) was preincubated for 10 min with the cholesteryl [9,10-^{3}H]oleate (20 nmol) before the reaction was initiated by the addition of a suspension of mammary gland microsomes (100 μg) in a solution of bovine serum albumin (see the Materials and Methods section). The results are means ± SEM for triplicate assays from two experiments. Abbreviation as in Figure 1.

against the water-soluble substrate *p*-nitrophenyl-butyrate was not affected by either enolase (results not shown).

Both enolase preparations were further purified by FPLC using a Superose-12 gel-filtration column. In each instance, the protein in the peak fraction, which had both enolase and inhibitory activity, showed only a single band when analyzed by PAGE in three different buffer systems (Figs. 3 and 4). Consistent with greater than 99% purity of the initial preparations, there was no change in either catalytic or inhibitory specific activity in these FPLC-purified fractions (results not shown). Nevertheless, the enolase catalytic activity was unrelated to the inhibitory activity, as the inclusion of 5 mM KF and 5 mM potassium phosphate completely inhibited enolase activity, whereas inhibitory activity due to 0.65 nmol of rab-

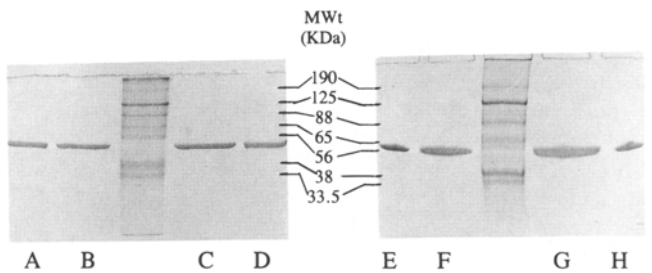


FIG. 3. SDS–polyacrylamide gel electrophoresis analysis of yeast and rabbit muscle enolase after purification by fast-performance liquid chromatography (FPLC) gel-filtration. Lanes A–D were performed on 10% discontinuous Laemmli gels (Ref. 16) and lanes E–H were performed on continuous 5.6% gels with the buffer system of Fairbanks *et al.* (Ref. 18). Each lane contained 5 μg protein and the gels were stained with Coomassie blue. Yeast enolase (i) before FPLC, lanes A and E, (ii) after FPLC, lanes B and F. Rabbit muscle enolase (iii) before FPLC, lanes D and H, (iv) after FPLC, lanes C and G.

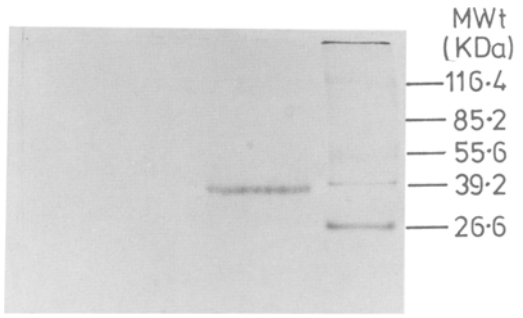


FIG. 4. Electrophoretic analysis of rabbit muscle enolase after FPLC gel-filtration. Electrophoresis was performed on discontinuous 10% acrylamide gels using the cationic detergent, tetradecyl trimethyl ammonium bromide, and the buffer system of Amory *et al.* (Ref. 17) at pH 2.0. The gel was stained with Coomassie blue after extensive washing to remove the detergent. Abbreviation as in Figure 3.

bit muscle enolase was reduced only 36% (from 31.8 ± 1.6 to 20.2 ± 4.4%) by 25 mM KF and 25 mM potassium phosphate. Moreover, the enolase catalytic activity was unaffected by HSL (results not shown).

Three peaks of enolase catalytic activity were obtained from the rat brain preparation, corresponding to the isoforms αα-, αγ-, and γγ- (15), and these coincided with three peaks of inhibitor activity (Fig. 5). The ratio of inhibition to enolase activity was, nevertheless, different in the three peak fractions, with the column fraction containing the αα-enolase (fraction 19) having the highest ratio and the fraction containing γγ-enolase (fraction 60) having the lowest (Table 1). The fraction containing the αγ-isoenzyme (fraction 40) was intermediate between these two extremes. This is consistent with the report that the α-isoenzyme of enolase was markedly increased in cholesterol-loaded macrophages (12).

Binding experiments. Although the bulk of the cholesteryl oleate was pelleted by centrifugation (Table 2), inclusion of BSA allowed retention of a greater proportion of the substrate in the supernatant (Table 2). Inclusion of an acetone–diethyl ether powder, prepared from the cytosolic fraction of rat liver, also increased retention in the supernatant, whereas the presence of either yeast or rabbit muscle enolase (0.56 nmol) increased the amount of cholesteryl ester that was pelleted, despite the fact that both are soluble proteins (Table 2). Neither the protein in the hepatic cytosolic fraction nor BSA (3.03 nmol), whether present in the initial incubation or added later, were able to prevent the precipitation of the cholesteryl ester by rabbit muscle enolase (Table 2). In contrast, the binding of cholesteryl oleate to the yeast enolase was much weaker, and BSA (3.03 nmol) effectively competed with this enolase, resulting in a greater amount being retained in the supernatant. Varying the amount of BSA present in the initial incubation indicated that the presence of even small amounts of BSA competed effectively with the yeast enolase (Fig. 6).

Although binding of cholesteryl oleate to rabbit muscle enolase varied with the concentration of the enolase, there were differences in the response depending on the presence of BSA (Table 2, Fig. 7). When BSA (3.03 nmol) was initially absent from the incubation, only a small amount of enolase

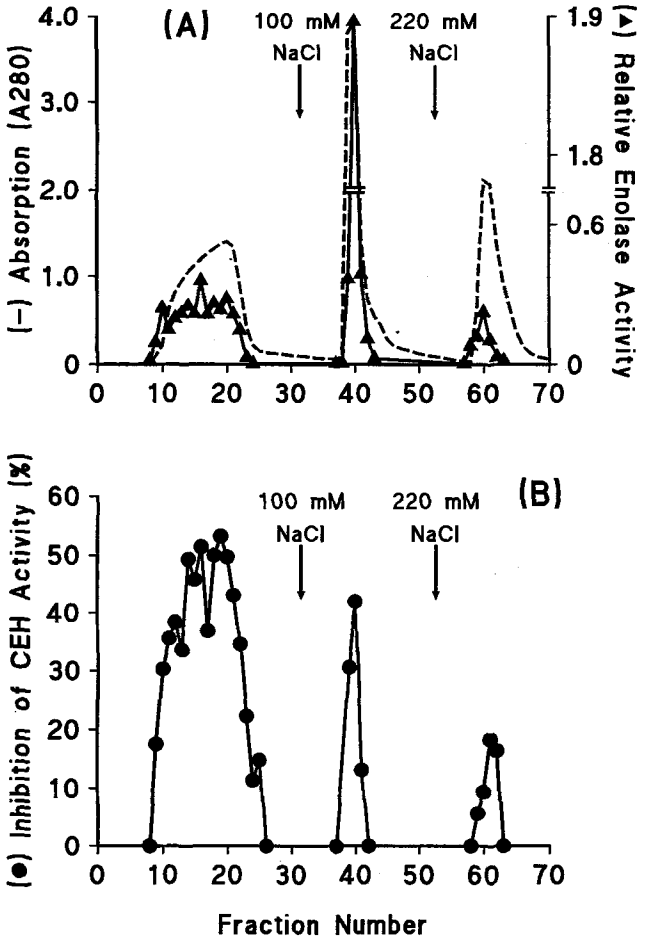


FIG. 5. Separation of enolase isoenzymes of rat brain by chromatography on Macro-prep Q. The column (2.5 × 5 cm) was initially eluted with homogenization buffer (15 mM TRIS–acetate, pH 6.5 containing 5 mM $MgSO_4$ and 0.1 mM EDTA), and this was changed to homogenization buffer containing either 100 mM NaCl or 220 mM NaCl at the points shown. Enolase activity (A) and CEH inhibitory activity (B) were determined as given in the Materials and Methods section. Abbreviation as in Figure 1.

TABLE 1
Relationship Between Inhibition of Neutral Cholesteryl Ester Hydrolase (CEH) and Enolase Activity for the Peak Fractions from the Macro-prep Q Column[a]

Fraction number	Protein (mg/mL)	Protein causing 25% inhibition of CEH (Xμg)	Equivalent enolase activity (nmol/min/Xμg)	Ratio (% inhibition/enolase activity)
19	1.34	20.3	14.6	1:1.712
40	4.06	30.5	104.3	1:0.239
60	2.31	687.6	362.9	1:0.069

[a]The three peak fractions, containing the different isoforms of enolase (fraction 19 = αα-isoform, fraction 40 = αγ-isoform, fraction 60 = γγ-isoform), from the Macro-prep Q column were assayed, in triplicate, for enolase activity and for inhibitory activity (against the mammary microsomal CEH). The amount of protein required to achieve 25% inhibition of enzyme activity was measured and the enolase activity equivalent to that amount of protein then calculated. From this data, the ratio of inhibition to enolase activity was determined.

TABLE 2
The Influence of Various Additions on the Retention of Cholesteryl Oleate in the Supernatant of Aqueous Media by Yeast and Rabbit Muscle Enolase[a]

Preincubation conditions	Second addition	Cholesteryl oleate in supernatant (nmol)
No additions	Cholesteryl oleate	2.22 ± 0.17
BSA	Cholesteryl oleate	16.74 ± 0.51
R.M. enolase	Cholesteryl oleate	0.40 ± 0.17
Yeast enolase	Cholesteryl oleate	0.28 ± 0.04
R.M. enolase + BSA	Cholesteryl oleate	1.1 ± 0.34
Yeast enolase + BSA	Cholesteryl oleate	16.28 ± 1.29
R.M. enolase + cholesteryl oleate	BSA	0.18 ± 0.06
Yeast enolase + cholesteryl oleate	BSA	0.19 ± 0.00
Acetone powder	Cholesteryl oleate	4.28 ± 0.15[b]
Acetone powder + enolase	Cholesteryl oleate	0.60 ± 0.10[b]

[a]The solutions contained various combinations of yeast and rabbit muscle (R.M.) enolase (0.55 nmol), bovine serum albumin (BSA) (3.03 nmol), and an acetone–diethyl ether powder obtained from rat liver cytosol (52.8 μg) in 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM TRIS, 0.5 mM EDTA buffer, pH 7.2. Cholesteryl [1-^{14}C]oleate (20 nmol, 108,000 dpm) was added as an ethanolic solution. The mixtures were preincubated at 37°C for 10 min before the second addition was made. Incubation was continued for a further 10 min before the tubes were cooled in an ice bath for 5 min prior to centrifugation (15,000 × *g*, 25 min). Duplicate aliquots of the supernatants were counted to determine the radioactivity remaining therein. The results shown are means ± SEM from at least triplicate assays.
[b]Determined using cholesteryl [9,10-^{3}H]oleate (20 nmol, 290,000 dpm).

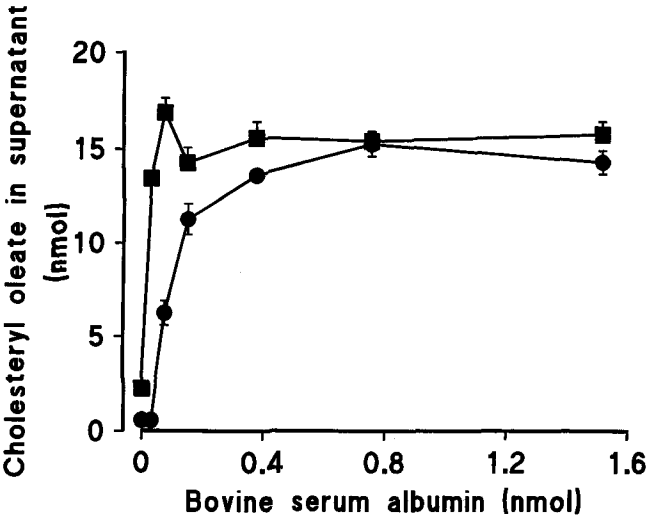


FIG. 6. Competition between yeast enolase and bovine serum albumin (BSA) for binding of cholesteryl oleate. Varying amounts of BSA were incubated at 37°C in 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM TRIS buffer, pH 7.2 (total volume 190 μL) initially in the absence (■) or the presence (●) of yeast enolase (0.56 nmol). After 10 min, an ethanolic solution (10 μL) of cholesteryl [1-^{14}C]oleate (20 nmol, 103,000 dpm) was added to the tubes, which were incubated for a further 10 min. The tubes were then cooled in ice (5 min) and centrifuged (15000 × *g*, 25 min) before duplicate aliquots of the supernatant were counted to determine the amount of cholesteryl ester remaining therein. The results are the means ± SEM for triplicate assays.

(16 pmol) was required to achieve precipitation of the bulk of the ester (Fig. 7A), and there was a good correlation (r = 0.86) between binding and inhibition of CEH activity (Fig. 8A). When the initial incubation was performed in the presence of BSA (3.03 nmol), much less of the cholesteryl ester was precipitated. Low enolase concentrations caused a slight increase in the amount of cholesteryl oleate retained in the supernatant, but precipitation of the substrate predominated at higher enolase concentrations (Fig. 7B). Due to this change from solubility to precipitation, the correlation between binding and inhibition (r = 0.66) was poorer (Fig. 8B).

These binding experiments thus suggested that the two enolases caused the inhibition by different mechanisms. This was confirmed by Lineweaver-Burk plots (Fig. 9), which indicated that the yeast enolase showed a "competitive" form of inhibition, whereas the plot exhibited by the muscle enolase was characteristic of an "uncompetitive," form of inhibition (23). Kinetic differences between mammalian and yeast enolases have been reported previously (24).

DISCUSSION

The results presented here indicate that, under the assay conditions used, the glycolytic enzyme enolase inhibited the hydrolysis of cholesteryl esters catalyzed by a number of different CEHs. The pattern of inhibition shown by the rabbit muscle enolase was comparable with the inhibition produced by the cytosolic fraction from rat liver (9), in terms of both the sensitivity of the different hydrolases to inhibition and the fact that total inhibition could not be obtained. Moreover, as with the hepatic inhibitory protein, the efficiency of inhibition was very dependent on the mode of initiation of the reaction.

This property was exhibited by rabbit muscle enolase, purchased from a number of different suppliers, as well as by the yeast enolase. This not only indicates that it is a feature of the enolase molecule but also diminishes the possibility that the inhibitory activity was due to an impurity co-purified with the enzyme. The results of PAGE–SDS analysis on three different systems, indicating the purity of the enzyme following gel-filtration by FPLC, also confirms that the inhibition was due to the enolase molecule (Figs. 3 and 4).

The binding experiments indicate that, although cholesteryl oleate is insoluble in aqueous media, binding to soluble proteins allows more of the ester to be retained in solution. Although this occurred with BSA and with the inhibitory material in the cytosolic fraction of rat liver (Table 2; Ref. 9), the binding of both yeast and rabbit muscle enolase caused aggregation and increased precipitation of the sterol ester (Fig. 7, Table 2). This is clearly a strong reaction with the rabbit muscle enolase as 16 pmol of the enzyme protein was able to cause aggregation of more than 4 nmol of the ester even when BSA (3 nmol) was added later as a competing protein (Fig. 7B). In contrast, the binding between the cholesteryl ester and yeast enolase was much weaker because as little as 0.35 nmol of BSA effectively prevented 0.56 nmol of the enolase from causing precipitation of the cholesteryl ester

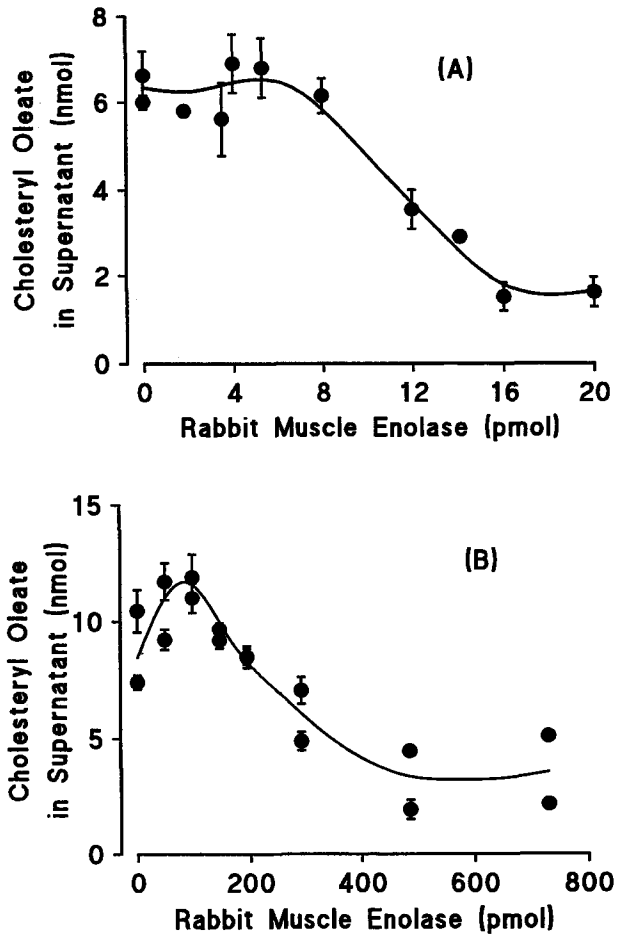


FIG. 7. Binding of cholesteryl ester to rabbit muscle enolase. Varying amounts of rabbit muscle enolase were incubated at 37°C in 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM TRIS buffer, pH 7.2, with cholesteryl [9,10-^{3}H]oleate (20 nmol, 250,000 dpm) either initially in the absence (A) or the presence (B) of BSA (3 nmol). After 10 min, an equivalent amount of BSA was added to the first tubes (final volume 200 μL), and both sets of tubes were incubated for another 10 min. The tubes were then cooled in ice (5 min) and centrifuged (15,000 × *g*, 25 min) before duplicate aliquots of the supernatant were counted to determine the amount of cholesteryl ester remaining therein. The results are the means ± SEM for triplicate assays from two separate experiments in each instance. Abbreviation as in Figure 6.

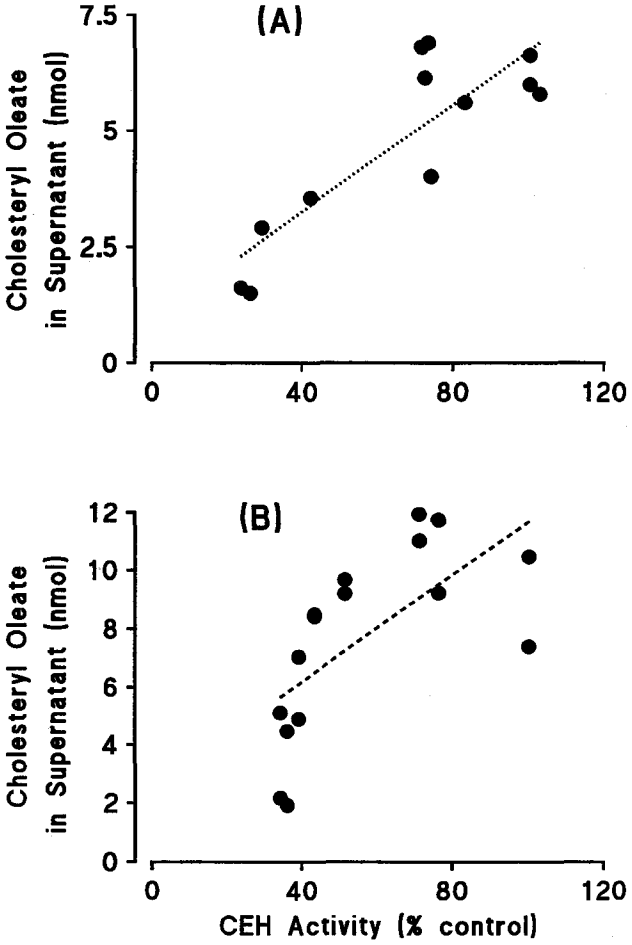


FIG. 8. Scatter diagram relating the binding of cholesteryl oleate and the inhibition of cholesteryl ester hydrolysis produced by rabbit muscle enolase in the initial absence (A), and presence (B) of BSA. The means for triplicate assays from two separate experiments are plotted. Linear regression analysis of the results in (A) gave y = 0.058(x) + 0.921, r = 0.86, and in (B) gave y = 0.92(x) + 2.52, r = 0.66. Abbreviations as in Figures 1 and 6.

(Fig. 6). Aggregation of the cholesteryl ester by the rabbit muscle enolase also occurred in the presence of an excess of liver cytosolic protein, suggesting that binding between the enolase and the cholesteryl ester could also occur in the cytosol *in vivo*.

Although the mechanism for the inhibition of CEH activity is presently unknown, the binding studies suggest that at least part of the process involves an interaction between substrate and enolase, which serves to protect the substrate from the hydrolytic activity of the enzyme. This interpretation is supported both by the binding experiments and by the inability of the enolases to inhibit the hydrolysis of the water-soluble *p*-nitrophenylbutyrate by HSL. Moreover, both the binding experiments and the Lineweaver-Burk plots indicate that there are differences in the mechanism of binding between the substrate and the two enolases, which is further influenced by the addition of other components to the mixture. This then could provide an explanation for the differential sensitivity of the various hydrolases to enolase inhibition. In particular, the liver and mammary gland microsomes are multicomponent systems which could influence the binding in a multitude of ways. The differential binding properties could also reflect the fact that, on average, there is only a 62% sequence identity between yeast and mammalian enolases (25).

The interest in this inhibitory property arises from the observation by Tabas and co-workers (12) that, when macrophages were loaded with cholesteryl esters and converted into "foam" cells, an isoform of α-enolase was increased 4–5-fold. However, cholesterol-loaded macrophages did not exhibit an increase in enolase glycolytic activity, implying that the increased form of α-enolase was not catalytically active, and thus might have some other function. Enolase is known to undergo interactions with other macromolecules and to have secondary functions in some cells (25). The

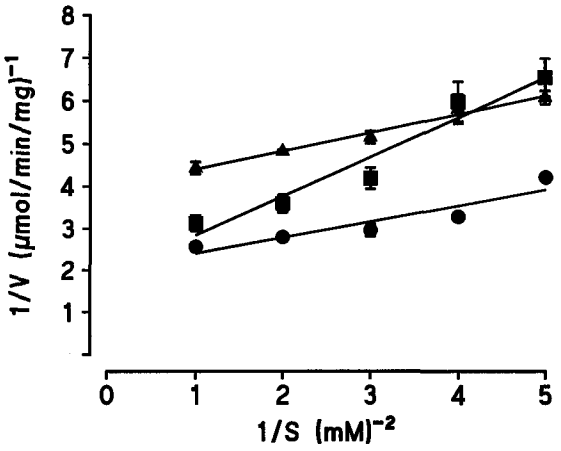


FIG. 9. Influence of yeast and rabbit muscle enolases on cholesteryl ester hydrolysis by hormone-sensitive lipase. Lineweaver-Burk plots of cholesteryl oleate hydrolysis by hormone-sensitive lipase at different substrate concentrations in the absence (●) or presence of either (■) yeast enolase (0.18 nmol) or (▲) rabbit muscle enolase (0.67 nmol).

results presented here suggest that such a secondary function in macrophages could be inhibition and, hence, control of CEH activity.

The observation that enolase acts to inhibit CEHs may also have far-reaching implications beyond the field of atherosclerosis. This glycolytic enzyme is particularly abundant and can constitute around 1% of soluble cell protein, notably in brain tissue (26). This may be relevant to some neuro-degenerative diseases in which increases in enolase activity have been correlated with increases in cholesteryl ester content. Thus, increases in α- and γ-isoenzymes of enolase, as well as accumulations of cholesteryl esters, have been detected in the brain tissue of sufferers of multiple sclerosis and Wallerian degeneration (27–30). The concentration of enolase is also increased in brain tissue of sufferers of transmissible spongiform encephalopathies, e.g., scrapie-affected mice and patients infected with Creuzfeldt Jacob's disease. Moreover, recent research has confirmed an increase in cholesteryl esters in such tissue (31). It is thus particularly interesting that partially purified enolase preparations from rat brain tissue exhibited these inhibitory properties.

In addition, cholesterol metabolism is altered in tumor cell proliferation, and increases in γ-enolase have been used as a diagnostic indicator in a number of cancerous conditions of neuroendocrine origin, particularly in small cell lung cancer (32,33). Thus, there is considerable evidence correlating cholesteryl ester accumulation with an increase in enolase protein, and the results presented here could provide an explanation for that correlation.

It has been speculated that the heterogeneity exhibited by enolases may confer some selective advantage upon the organism. Control of cholesteryl ester hydrolysis may be one such advantage, which may provide a new target for the treatment of those diseases in which cholesterol metabolism is altered.

ACKNOWLEDGMENTS

We thank Professor S.J. Yeaman for the gift of the hormone-sensitive lipase. This research was funded by the Scottish Office Agriculture and Fisheries Department.

REFERENCES

1. Khoo, J.C., Mahoney, E.M., and Steinberg, D. (1981) Neutral Cholesteryl Esterase Activity in Macrophages and Its Enhancement by a cAMP-Dependent Protein Kinase, *J. Biol. Chem. 256,* 12659–12661.
2. Tabas, I., Weiland, D.A., and Tall, A.R. (1985) Unmodified Low Density Lipoprotein Causes Cholesteryl Ester Accumulation in J774 Macrophages, *Proc. Natl. Acad. Sci. USA 82,* 416–420.
3. Tabas, I., Roscoff, W.J., and Boykow, G.C. (1988) Acyl CoA:Cholesterol Acyl Transferase in Macrophages Utilizes a Cellular Pool of Cholesterol Oxidase-Accessible Cholesterol as Substrate, *J. Biol. Chem. 263,* 1266–1272.
4. Brown, M.S., Ho, Y.K., and Goldstein, J.L. (1980) The Cholesteryl Ester Cycle in Macrophages: Continual Hydrolysis and Reesterification of Cytoplasmic Cholesteryl Esters, *J. Biol. Chem. 255,* 9344–9352.
5. Brown, M.S., and Goldstein, J.L. (1983) Lipoprotein Metabolism in Macrophages: Implications for Cholesterol Deposition in Atherosclerosis, *Ann. Rev. Biochem. 52,* 223–261.
6. Gerrity, R.G. (1981) The Role of the Monocyte in Atherogenesis. I. Transition of Blood-Borne Monocytes into Foam Cells in Fatty Lesions, *Am. J. Pathol. 103,* 181–190.
7. Faggioto, A., Ross, R., and Harker, L. (1984) Studies of Hypercholesterolemia in Nonhuman Primates. I. Changes That Lead to Fatty Streak Formation, *Arteriosclerosis 4,* 323–340.
8. Raines, E.W., and Ross, R. (1989) in *Human Monocytes* (Zembala, M. and Asherson, G., eds.) pp. 247–259, Academic Press, London.
9. Shand, J.H., and West, D.W. (1992) Characterization of a Cytosolic Protein in Rat Liver Inhibiting Neutral Cholesteryl Ester Hydrolase, *Lipids 27,* 406–412.
10. Shand, J.H., Crilly, P.J., and West, D.W. (1993) Inhibition of Neutral Cholesteryl Ester Hydrolase by a Naturally Occurring Cytosolic Protein in Macrophages, *FEBS Lett. 321,* 132–134.
11. Shand, J.H., and West, D.W. (1992) The Inhibition of Neutral Cholesteryl Ester Hydrolase by a Cytosolic Protein Factor in Female Rat Liver: The Influence of Varying Hormonal and Nutritional Conditions on the Inhibitory Activity, *Lipids 27,* 417–417.
12. Bottalico, L.A., Kendrick, N.C., Keller, A., Li, Y., and Tabas, I. (1993) Cholesteryl Ester Loading of Mouse Peritoneal Macrophages Is Associated with Changes in the Expression or Modification of Specific Cellular Proteins, Including Increase in an α-Enolase Isoform, *Arteriosclerosis and Thrombosis 13,* 264–275.
13. Rider, C.C., and Taylor, C.B. (1975) Enolase Isoenzymes: II Hybridization Studies, Developmental and Phylogenetic Aspects, *Biochim. Biophys. Acta 405,* 175–187.
14. Keller, A., Scarna, H., Mermet, A., and Pujol, J-F. (1981) Biochemical and Immunological Properties of the Mouse Brain Enolases Purified by a Simple Method, *J. Neurochem. 36,* 1389–1397.
15. Suzuki, F., Umeda, Y., and Kato, K. (1980) Rat Brain Enolase Isozymes. Purification of Three Forms of Enolase, *J. Biochem. 87,* 1587–1594.
16. Laemmli, U.K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, *Nature 227,* 680–685.

17. Amory, A., Foury, F., and Goffeau, A. (1980) The Purified Plasma Membrane ATPase of the Yeast *Schizosaccharomyces pombe* Forms a Phosphorylated Intermediate, *J. Biol. Chem. 255,* 9353–9357.
18. Fairbanks, G., Streck, T.L., and Wallace, D.F.H. (1971) Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocyte Membrane, *Biochemistry 10,* 2606–2617.
19. Bradford, M. (1976) A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72,* 248–254.
20. Small, C.A., Yeaman, S.J., West, D.W., and Clegg, R.A. (1991) Cholesteryl Ester Hydrolase and Hormone-Sensitive Lipase in Lactating Rat Mammary Tissue, *Biochim. Biophys. Acta 1082,* 251–254.
21. Chen, J.J.S., Rothman, V., and Margolis, S. (1986) Evidence That Cholesteryl Ester Hydrolase and Triglyceride Lipase Are Different Enzymes in Rat Liver, *Lipids 21,* 503–507.
22. Ghosh, S., and Grogan, W.M. (1991) Rapid Three-Step Purification of a Hepatic Neutral Cholesteryl Ester Hydrolase Which Is Not the Pancreatic Enzyme, *Lipids 26,* 793–798.
23. Dixon, M., Webb, E.C., Thorne, C.J.R., and Tipton, K.F. (1979) *Enzymes,* 3rd edn., pp. 332–381, Longman, London.
24. Kornblatt, M.J., and Klugerman, A. (1989) Characterization of the Enolase Isozymes of Rabbit Brain: Kinetic Differences Between Mammalian and Yeast Enolases, *Biochem. Cell Biol. 67,* 103–107.
25. Lebioda, L., and Stec, B. (1991) Mapping of Isozymic Differences in Enolase, *Int. J. Biol. Macromol. 13,* 97–100.
26. Kato, K., Suzuki, F., and Umeda, Y. (1981) Highly Sensitive Immunoassays for Rat Brain Enolase Isozymes, *J. Neurochem. 36,* 793–797.
27. Green, J.B., Papadopoulos, N., Cevallos, W., Forster, F.M., and Hess, W.C. (1959) The Cholesterol and Cholesterol Ester Content of Cerebrospinal Fluid in Patients with Multiple Sclerosis and Other Neurological Diseases, *J. Neurol. Neurosurg. Psychiat. 22,* 117–119.
28. Mezei, C. (1970) Cholesteryl Esters and Hydrolytic Cholesterol Esterase During Wallerian Degradation, *J. Neurochem. 17,* 1163–1170.
29. Wender, M., Filipek, H., and Stanislawska, B. (1974) Cholesterol Esters of the Rat Brain in Demyelinating Diseases, *Clin. Chim. Acta. 54,* 269–275.
30. Ghosh, S., and Grogan, W.M. (1991) Decline in Cholesteryl Ester Hydrolase Activity of Rat Brain with Progress of Experimental Allergic Encephalomyelitis Followed by Rebound During Recovery, *Brain Research 547,* 327–330.
31. Perring, T.D.M., Hope, J., and Baker, R.L. (1993) Detection of Aberrant Cerebral Cholesteryl Ester Accumulation in a Mouse Model of Scrapie, *Biochem. Soc. Trans.*, in press.
32. Fujita, K., Haimoto, H., Imaizumi, M., Abe, T., and Kato, K. (1987) Evaluation of Gamma Enolase as a Tumor Marker for Lung Cancer, *Cancer 60,* 362–369.
33. Jorgensen, L.G.M., Hansen, H.H., and Cooper, E.H. (1989) Neuron-Specific Enolase, Carcinoembryonic Antigen and Lactate Dehydrogenase as Indicators of Disease Activity in Small Cell Lung Cancer, *Eur. J. Cancer Clin. Oncol. 25,* 123–128.

[Received September 16, 1994, and in revised form May 4, 1995; Revision accepted May 15, 1995]

Inhibition of Cholesterol Esterification in Macrophages and Vascular Smooth Muscle Foam Cells: Evaluation of E5324, an Acyl–CoA Cholesterol Acyltransferase Inhibitor

Andrew C. Nicholson, Kenneth B. Pomerantz, Tohru Fujimori and David P. Hajjar*

Departments of Pathology and Biochemistry, Cornell University Medical College, New York, New York 10021

ABSTRACT: Cholesteryl esters (CE) comprise the principal lipid class that accumulates within macrophages and smooth muscle cells of the atherosclerotic lesion. Acyl–CoA cholesterol acyltransferase (ACAT) is the major enzyme responsible for esterification of intracellular cholesterol. We evaluated the ability of E5324 (*n*-butyl-*N'*-[-2-[3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy]-6-methyl-phenyl]urea), a novel, orally absorbable ACAT inhibitor, to inhibit esterification of fatty acids to cholesterol and CE accumulation in macrophages and in smooth muscle cells. E5324 significantly inhibited cholesterol esterification in rat aortic smooth muscle cells and in macrophages. In addition, E5324 reduced the cellular mass of CE, the significant measure of the efficacy of drugs designed to modulate cholesterol metabolism. E5324 treatment of macrophages exposed to acetylated low-density lipoprotein reduced CE mass by 97%, and treatment of lipid-loaded smooth muscle cells reduced CE mass by 29%. Although free cholesterol increased approximately twofold, this free cholesterol would presumably be accessible to the membrane for efflux *in vivo* (reverse cholesterol transport). These results demonstrate that E5324 can inhibit cholesterol esterification and CE mass in atherosclerotic foam cells, derived from either macrophages or arterial smooth muscle cells.

Lipids 30, 771–774 (1995).

Atherosclerosis and its associated pathological processes (coronary heart disease, peripheral vascular disease, and stroke) are major causes of morbidity and mortality in industrialized nations. Epidemiological studies have established a link between serum cholesterol levels and the development of atherosclerosis, and have demonstrated that lowering serum cholesterol levels reduces the risk of coronary heart disease. The level of serum cholesterol is determined by the absorption of dietary cholesterol, endogenous synthesis of cholesterol, and excretion of cholesterol into the bile. Each of these areas represents a site for potential therapeutic intervention to lower total serum cholesterol. Cholestyramine, a resin which combines with bile acids in the intestine, has been used successfully to prevent reabsorption of cholesterol and enhance its excretion. Inhibitors of hydroxymethylglutaryl–CoA reductase reduce endogenous synthesis of cholesterol and are widely used clinically.

Inhibition of acyl–CoA cholesterol acyltransferase (ACAT) is also a potential site of modulation of cholesterol and cholesteryl ester (CE) accumulation in atherosclerosis (1). ACAT is a microsomal enzyme which catalyzes the intracellular esterification of cholesterol to a free fatty acid, resulting in the formation of CE, the principal storage form of cholesterol (2). ACAT activity is increased within atherosclerotic lesions in animals with experimental atherosclerosis (3–5). ACAT inhibitors could have beneficial effects in treating or preventing atherosclerosis by reducing absorption of dietary cholesterol or inhibiting production of lipoproteins by the liver. ACAT inhibitors could also inhibit the accumulation of CE within macrophages and smooth muscle cells of the atheroma, facilitating the efflux of cholesterol and inhibiting the progression of atherosclerosis.

A novel ACAT inhibitor, E5324, was discovered after an intensive screening program (6,7), and was reported to have an inhibitory effect on ACAT activity in microsomes from various tissues, as well as in cultured cell homogenates (8). It also had anti-atherosclerotic activity in a rabbit model of atherosclerosis (9). We evaluated the effects of E5324 on the inhibition cholesterol esterification and on accumulation of cholesterol and CE in vascular smooth muscle cells and macrophages, the two cell types which accumulate CE and become the characteristic foam cells of the atherosclerotic lesion (10,11). We also evaluated the effects of E5324 on the two major enzymes in smooth muscle cells responsible for CE hydrolysis—lysosomal (acid) CE hydrolase and cytoplasmic (neutral) CE hydrolase. Lastly, the ability of E5324 to alter cholesterol and CE mass was evaluated in smooth muscle cells exposed to cationized low-density lipoprotein (LDL), an *in vitro* model of smooth muscle cell foam cell development.

EXPERIMENTAL PROCEDURES

Materials. E5324 (*n*-butyl-*N'*-[-2-[3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy]-6-methyl-phenyl]urea) was a generous gift from the Tsukuba Research Laboratories of Eisai Co. Ltd. (Ibaraki, Japan). Human plasma LDL (d = 1.019–1.063

*To whom correspondence should be addressed at Dept. of Pathology, Cornell University Medical College, 1300 York Ave., New York, NY 10021.

Abbreviations: ACAT, acyl–CoA cholesterol acyltransferase; ACEH, acid cholesteryl ester hydrolase; CE, cholesteryl ester; LDL, low-density lipoprotein; NCEH, neutral cholesteryl ester hydrolase; PBS, phosphate-buffered saline.

Copyright © 1995 by AOCS Press

g/mL) was isolated by ultracentrifugation (12). The LDL was dialyzed against HEPES-buffered saline with EDTA, sterilized by filtration through a 0.22 μm filter, and stored under nitrogen gas at 4°C. Acetylation of LDL was described by Goldstein *et al.* (13). Cationized LDL was made by the method of Basu *et al.* (14,15).

Cholesterol esterification assay. Esterification of cholesterol was measured as described by Brown *et al.* (16). Substrate (LDL or acetylated LDL) was added with [^{14}C]-oleate (final conc. 0.1 mM oleate, 0.02 mM albumin). Cellular lipids were extracted into hexane/isopropanol (3:2) and spotted on thin-layer chromatography plates (Silica Gel 60; Analtech, Newark, DE). Areas corresponding to CE were scraped and counted in a Beckman scintillation counter (Beckman Instruments, Fullerton, CA).

Acid cholesteryl ester hydrolase (ACEH) and neutral cholesteryl ester hydrolase (NCEH) assays. ACEH (17) and NCEH (18) activity of cell homogenates were performed as previously described.

Cells and cell lines. J774 macrophages, a murine macrophage cell line, were obtained from American Type Culture Collection (ATCC, Rockville, MD). They were subpassaged in RPMI 1640 with 10% fetal bovine serum. Smooth muscle cells were propagated from explants of rat thoracic aorta in Medium 199 (Gibco BRL; Life Technologies Inc., Gathersburg, MD) containing glutamine, penicillin/streptomycin, Fungizone (Flow Laboratories, Inc., Mclean, VA), and 10% fetal bovine serum.

Cholesterol and CE mass. Cholesterol and CE mass were quantified by gas–liquid chromatography. Lipids were extracted as described by Brown *et al.* (16). The cell lipid extract was divided into two portions. The first was analyzed directly for determination of free cholesterol and the second was hydrolyzed using methanolic potassium hydroxide, followed by re-extraction into hexane and gas–chromatographic analysis for measurement of total cholesterol. The sterols were separated using a Hewlett-Packard (Palo Alto, CA) 5880A gas chromatograph on a six-foot OV-17 WHP column (Supelco, Bellefonte, PA), at a nitrogen flow rate of 40 mL/min, using a thermal gradient program starting at 260°C and raised 1°C/min to a final temperature of 275°C (19). The quantity of cholesterol (and CE) was determined by proportional area computation of mass/peak ratios toward known amounts of added β-sitosterol. Results were normalized to cellular protein levels measured by the method of Lowry *et al.* (20).

Statistical analysis. Values are expressed as mean ± SEM. Significance of the mean values were determined by the Student's *t*-test ($P < 0.05$).

RESULTS

We first examined the dose response relationship of E5324 to inhibit cholesterol esterification in smooth muscle cells. E5324 significantly inhibited cholesterol esterification at concentrations of 1 μM (30% reduction) and 10 μM (67% reduction) (Fig. 1). E5324 (10 μM) also inhibited esterification in macrophages by 73% (Fig. 2). As E5324 significantly inhibited cholesterol esterification in macrophages, we next evaluated whether E-5324 could alter cholesterol or CE mass in macrophages incubated with acetylated LDL, a scavenger receptor ligand. Incubation of macrophages with acetylated LDL, but not native LDL, results in significant accumulation of CE (13). Treatment of macrophages exposed to acetylated LDL with E5324 significantly reduced CE mass (by 93% relative to macrophages exposed to acetylated LDL alone) (Fig. 3). Free cholesterol increased approximately twofold. E5324 had no significant effects on ACEH or NCEH activities.

Because foam cells of smooth muscle origin are a prominent component of the atherosclerotic lesion, we utilized an *in vitro* model of smooth muscle foam cells. Smooth muscle cells were incubated with cationized LDL to evaluate the potential modulatory effect of E-5324 on cholesterol and CE mass in smooth muscle foam cells. This charge-modified LDL is internalized in a nonreceptor mediated manner by bulk phase endocytosis, resulting in marked CE accumulation (19,21). Incubation with native LDL or acetylated LDL will not result in lipid loading of smooth muscle cells. Treatment of CE-enriched smooth muscle cells with E5324 significantly

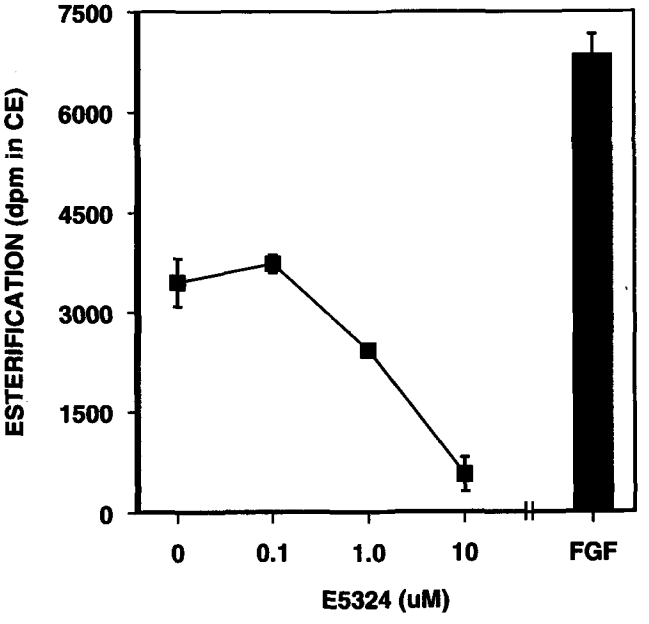


FIG. 1. Dose response of E5324 (Tsukuba Research Laboratories of Esai Co. Ltd., Ibaraki, Japan) on cholesterol esterification by rat aortic smooth muscle cells. Rat aortic smooth muscle cells were grown to confluence and incubated overnight in serum-free medium. Basic FGF (1 ng/mL) was added to one group as positive control. After 19 h, the cells (except for the bFGF-treated cells) were washed in phosphate-buffered saline and incubated for 5 h with media containing 0, 0.1 μM, 1.0 μM, and 10.0 μM E-5324, 50 μg/mL of LDL (cholesterol substrate), and [^{14}C]-oleate (final conc. 0.1 mM oleate, 0.02 mM albumin). Cellular lipids were separated in thin-layer chromatography and counted in a Beckman scintillation counter (Beckman Instruments, Fullerton, CA). The data represent the mean of three wells ± SEM. CE, cholesteryl ester; bFGF, basic fibroblast growth facter.

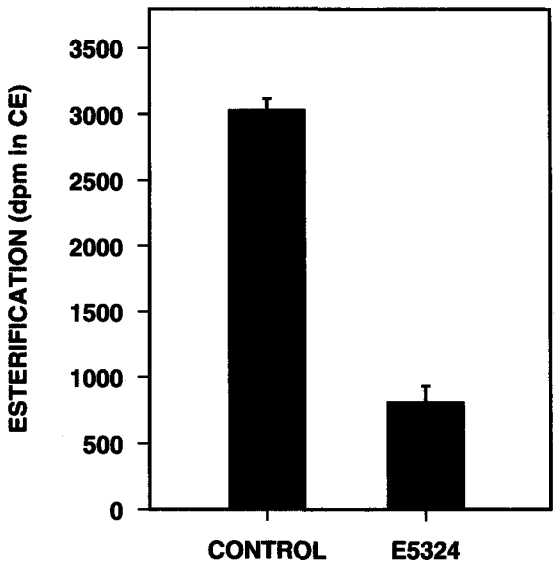


FIG. 2. Effect of E5324 on cholesterol esterification in macrophages. J774 macrophages, a murine macrophage cell line, were grown to confluence in RPMI 1640 with 10% fetal bovine serum in six-well plates. Incubation times are as described in Figure 1. Extraction of lipids and thin-layer chromatography procedures were as described in the Experimental Procedures section, with 10 μM E5324 utilized in the experimental group. The data represent the mean ± SEM of three wells. Abbreviation as in Figure 1, as well as company source.

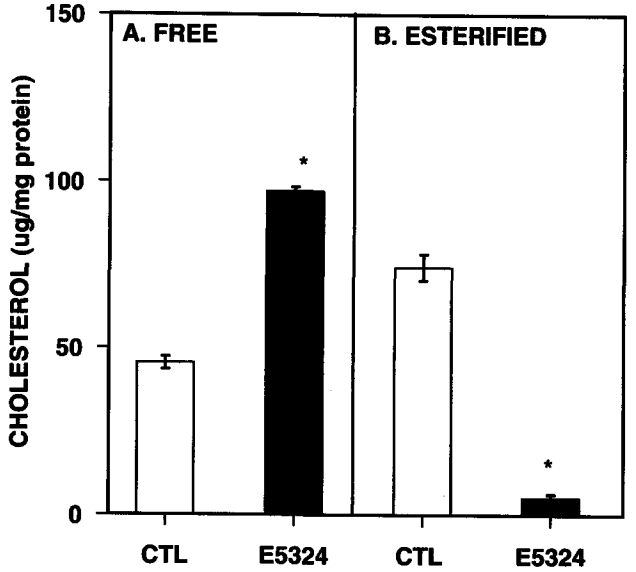


FIG. 3. Effect of E5324 on cholesterol and CE mass in macrophages. J774 macrophages were grown to confluence in 25 cm^2 flasks in RPMI with 10% fetal bovine serum. The media was changed to serum-free RPMI with 100 μg/mL acetylated low-density lipoprotein ± 10 μM E5324. The cells were incubated for 24 h prior to washing at 4°C three times with phosphate-buffered saline (PBS) with albumin and three times with PBS alone, and extraction of lipids in hexane/isopropanol (3:2). β-Sitosterol (30 μg) was added to each flask as internal standard. The cells were solubilized in 1 mL 0.2% NaOH for protein determination. The data represent the mean ± SEM of four flasks. *, $P < 0.05$ vs. control (CTL). See Figure 1 for company source.

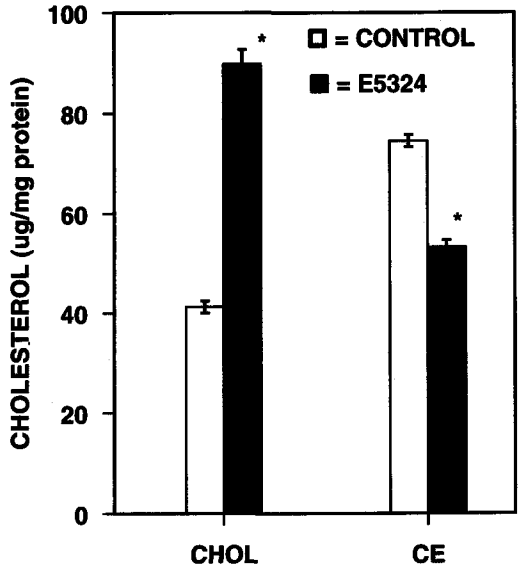


FIG. 4. Effect of E5324 on cholesterol (CHOL) and cholesteryl ester (CE) mass in smooth muscle cell foam cells. Rat aortic smooth muscle cells were exposed to cationized low-density lipoprotein (50 μg/mL) for 24 h in media with 5% fetal bovine serum. After loading, the media was changed and flasks were divided into two groups in triplicate; serum-free media alone and serum-free media with 10 μM E5324. The cells were incubated for 24 h prior to washing and extraction of lipids. β-Sitosterol (30 μg) was added to each flask as internal standard. The cells were solubilized in 0.2% NaOH for protein determination. Cholesterol and CE mass were quantified by gas–liquid chromatography. The data represent the mean ± SEM of three flasks. *, $P < 0.05$ vs. control. See Figure 1 for company source.

reduced CE mass (by 29%). Free cholesterol increased approximately 2.2-fold (Fig. 4).

DISCUSSION

Previous reports have shown that E5324, when administered concurrently with a high-cholesterol diet in rabbits, will prevent increases in plasma lipids and atherosclerotic plaque development (9). Furthermore, in pre-established hypercholesterolemic rabbits, E5324 can also lower plasma lipid levels, reduce plaque thickness, and reduce cholesterol accumulation within atherosclerotic plaques. E5324 also suppressed the accumulation of CEs in arterial lesions independently of its plasma cholesterol lowering effect (9). As these studies implied that E5324 has a direct effect on cells residing within the arterial wall, the current studies were undertaken to determine if E5324 altered cholesterol esterification *in vitro* in smooth muscle cells and macrophages, the progenitor cells for atherosclerotic foam cells.

E5324 significantly inhibited cholesterol esterification in aortic smooth muscle cells and macrophages. In addition, E5324 reduced the cellular mass of CE, a measure of the efficacy of drugs designed to modulate atherosclerotic lesion development. E5324 treatment of macrophages exposed to acetylated LDL reduced CE mass by 93%. Treatment of

"lipid-loaded" smooth muscle cells with E5324 reduced CE mass by 29%. Although free cholesterol increased approximately twofold in both macrophages and in smooth muscle cells, this free cholesterol would, presumably, be accessible to the membrane for efflux (reverse cholesterol transport) *in vivo*.

ACAT inhibitors have been studied for more than ten years, and a diverse set of compounds have been shown to be efficacious in lowering serum cholesterol levels in animal models of hypercholesterolemia. ACAT inhibitors also can decrease atherosclerotic lesion size and cholesterol accumulation in cholesterol-fed animals, but it is unclear if these effects are the indirect result of reduced serum cholesterol levels or a direct result of ACAT inhibition with macrophages and smooth muscle cells within the lesion. In addition to E5324 (9), one other ACAT inhibitor, CI-976, has been shown to inhibit CE accumulation within atherosclerotic arteries independent of lipid-lowering effects (22).

In summary, our data demonstrate that E5324 is effective in inhibiting cholesterol esterification in vascular smooth muscle cells and in macrophages. An important point is that E5324 also has the capacity to inhibit esterification and CE mass in smooth muscle foam cells which are already lipid-laden. These results are particularly significant as previous experments have shown that ACAT inhibitors can inhibit esterification only during the process of lipid loading.

REFERENCES

1. Sliskovic, D., and White, A. (1991) Therapeutic Potential of ACAT Inhibitors as Lipid Lowering and Anti-Atherosclerotic Agents, *Trends Pharm. Sci. 12*, 194–199.
2. Suckling, K., and Stange, E. (1985) Role of Acyl–CoA:Cholesterol Acyltransferase in Cellular Cholesterol Metabolism, *J. Lipid Res. 26*, 647–671.
3. Gillies, P., Rathgeb, K., Perri, M., and Robinson, C. (1986) Regulation of Acyl–CoA:Cholesterol Acyltransferase Activity in Normal and Atherosclerotic Rabbits: Role of a Cholesterol Substrate Pool, *Expt. Mol. Pathol. 44*, 329–339.
4. Hashimoto, S., and Dayton, S. (1975) Influence of Microsomal Cholesterol Concentrations on the Cholesterol-Esterifying Activity of Normal and Atherosclerotic Aortas, *Artery 1*, 308–318.
5. Brecher, P., and Chobanian, A. (1974) Cholesteryl Ester Synthesis in Normal and Atherosclerotic Aortas of Rabbit and Rhesus Monkeys, *Circ. Res. 35*, 692–701.
6. Kimura, T., Takase, Y., Hayashi, K., Tanaka, H., Ohtsuka, I., Saeki, T., Kogushi, M., Yamada, T., Fujimori, T., Saitou, I., and Akasaka, K. (1993) Structure-Activity Relationship of *N*-[2-(dimethylamino)-6-[3-(5-methyl-4-phenyl-1*H*-imidazol-1-yl)propoxy]-*N'*-pentylurea and Analogues. Novel Potent Inhibitors of Acyl–CoA:Cholesterol *O*-Acyltransferase with Antiatherosclerotic Activity, *J. Med. Chem. 36*, 1630–1640.
7. Kimura, T., Watanabe, N., Matsui, M., Hayashi, K., Tanaka, K., Ohtsuka, I., Saeki, T., Kogushi, M., Kabayashi, H., Akasaka, K., Yamagishi, Y., Saitou, I., and Yamatsu, I. (1993) Structure-Activity Relationship of a Series of Phenylureas Linked to 4-Phenylimidazole. Novel Potent Inhibitors of Acyl–CoA:Cholesterol *O*-Acyltransferase with Antiatherosclerotic Activity 2, *J. Med. Chem. 36*, 1641–1653.
8. Kogushi, M., Ohtsuka, I., Tanaka, H., Kimura, T., Hayashi, K., Saito, I., and Yamatsu, I. (1992) I. E5324: A Potent and Novel Inhibitor of Acyl–CoA:Cholesterol Acyltransferase, in *Proceedings of the XI International Symposium on Drugs Affecting Lipid Metabolism*, p. 88 (abstract) Kluwer Academic Publishers, The Netherlands.
9. Tanaka, H., Ohtsuka, I., Kogushi, M., Kimura, T., Fugimori, T., Saeki, T., Hayashi, K., Yamada, T., Hiyoshi, H., and Saito, I. (1994) Effect of the Acyl–CoA:Cholesterol Acyltransferase Inhibitor, E5324, on Experimental Atherosclerosis in Rabbits, *Athero. 107*, 187–201.
10. Katsuda, S., Boyd, H., Fligner, C., Ross, R., and Gown, A. (1992) Human Atherosclerosis III. Immunocytochemical Analysis of the Cell Composition in Young Adults, *Amer. J. Pathol. 140*, 907–914 (1992).
11. Brown, M., and Goldstein, J. (1983) Lipoprotein Metabolism in the Macrophage: Implications for Cholesterol Deposition in Atherosclerosis, *Ann. Rev. Biochem. 52*, 223–261.
12. Havel, R., Eder, H., and Bradgon, J. (1955) The Distribution and Chemical Preparation of Ultracentrifugally Separated Lipoproteins in Human Serum, *J. Clin. Invest. 34*, 1345–1353.
13. Goldstein, J., Ho, Y., Basu, S., and Brown, M. (1979) Binding Site on Macrophages That Mediates the Uptake and Degradation of Acetylated Low Density Lipoprotein Producing Massive. Cholesterol Deposition, *Proc. Natl. Acad. Sci. USA 76*, 333–337
14. Basu, S., Anderson, R., Goldstein, J., and Brown, M. (1977) Metabolism of Cationized Lipoproteins by Human Fibroblasts. Biochemical and Morphologic Correlations, *J. Cell. Biol. 74*, 119–135.
15. Basu, S., Goldstein, J., Anderson, R., and Brown, M. (1976) Degradation of Cationized Low Density Lipoprotein and Regulation of Cholesterol Metabolism in Homozygous Familial Hypercholesterolemia, *Proc. Natl. Acad. Sci. USA 73*, 3178–3182.
16. Brown, M., Ho, Y., and Goldstein, J. (1980) The Cholesteryl Ester Cycle in Macrophage Foam Cells: Continual Hydrolysis and Re-esterification of Cytoplasmic Cholesteryl Esters, *J. Biol. Chem. 255*, 9344–9352.
17. Haley, N., Fowler, S., and deDuve, C. (1980) Lysosomal Acid Cholesteryl Esterase Activity in Normal and Lipid-laden Aortic Cells, *J. Lipid Res. 21*, 961–969.
18. Hajjar, D. (1986) Regulation of Neutral Cholesteryl Esterase in Arterial Smooth Muscle Cells: Stimulation by Agonists of Adenylate Cyclase and Cyclic Amp-dependent Protein Kinase, *Arch. Biochem. Biophys. 247*, 49–56.
19. Pomerantz, K., and Hajjar, D. (1989) Eicosanoid Metabolism in Cholesterol-Enriched Arterial Smooth Muscle Cells: Reduced Arachidonate Release with Concommitant Decrease in Cyclooxygenase Products, *J. Lipid Res. 30*, 1219–1231.
20. Lowry, O., Rosebrogh, N., Farr, A., and Randall, J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
21. Goldstein, J., Anderson, R., Buja, L., Basu, S., and Brown, M. (1977) Overloading Human Aortic Smooth Muscle Cells with Low Density Lipoprotein-Cholesteryl Esters Reproduces Features of Atherosclerosis *in vitro*, *J. Clin. Invest. 59*, 1196–1202.
22. Bocan, T., Mueller, S., Uhlendorf, P., Newton, R., and Krause, B. (1991) Comparison of CI-976, an ACAT Inhibitor, and Selected Lipid-Lowering Agents for Antiatherosclerotic Activity in Iliac-Femoral and Thoracic Aortic Lesions, *Arterioscler. Thromb. 11*, 1830–1843.

[Received March 20, 1995, and in final revised form May 25, 1995; Revision accepted May 30, 1995]

Tissue-Selective Inhibition of Sterol Synthesis in Mice by Pravastatin Sodium After a Single or Repeated Oral Administrations

Teiichiro Koga*, Takashi Kikuchi, Atsuhiro Miyazaki and Hiroyuki Koike

Pharmacology and Molecular Biology Research Laboratories, Sankyo, Co., Ltd., Tokyo 140, Japan

ABSTRACT: Pravastatin, an inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase, exhibits liver-selectivity in inhibiting sterol synthesis, when administered as a single oral dose to mice or rats, whereas lovastatin and simvastatin do not. This may be due to the fact that pravastatin is distributed intracellularly, to a large extent, in the liver and extracellularly in nonhepatic tissues. In the present study, we examined whether the difference in liver-selectivity among these three HMG–CoA reductase inhibitors observed in single-dose studies was preserved after repeated oral administrations of drugs to mice. *De novo* sterol synthesis in different tissues of mice was examined *in vivo* three hours after the last dose of drug by measuring incorporation of intraperitoneally injected [^{14}C]acetate into total sterols. Pravastatin administered orally for 11 consecutive days at 5 and 10 mg/kg exhibited a greater liver-selectivity than lovastatin and simvastatin: sterol synthesis was inhibited more than 60% in the liver by all three drugs, whereas that in nonhepatic tissues was inhibited less than 10% by pravastatin and more than 30% by lovastatin and simvastatinin in most of the nonhepatic tissues examined. Pravastatin administered orally for 11 consecutive days at 10 mg/kg caused more selective inhibition of sterol synthesis in liver *ex vivo* than two other inhibitors at the same dose. Pravastatin inhibited *de novo* sterol synthesis from [^{14}C]acetate into sterol fraction in the liver slices *in vitro*, but minimally in those of the spleen and testis, whereas lovastatin and simvastatin inhibited in those of all three tissues. Since the drug concentrations determined in the same tissue samples of the liver, spleen, and testis were almost comparable among the three drugs, it was suggested that the cellular distribution profiles of pravastatin observed in a single-dose study were preserved in the multiple-dose study. We conclude that the difference in tissue-selectivity between pravastatin and the other two inhibitors to inhibit sterol synthesis in mice is maintained, regardless of the duration of administration.
Lipids 30, 775-779 (1995).

Pravastatin administered as the sodium salt (1–3) is a potent and specific hydrophilic inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase, a key enzyme of sterol synthesis (4). It has been shown that pravastatin exhibits hepatocyte-selective inhibition of sterol synthesis (3,5,6), which is due to the selective uptake of this agent by hepatocytes through the carrier-mediated pathway (7). The drug also exhibited liver-selective inhibition of sterol synthesis when orally administered to mice or rats (3,5,6). Our recent study has demonstrated (6) that, after oral administration to rats, pravastatin is distributed mostly in the intracellular space in the liver and in the extracellular spaces in nonhepatic tissues. These cellular distribution profiles of pravastatin seem to underlie the liver-selective inhibition of sterol synthesis.

In contrast to pravastatin, lovastatin, and simvastatin administered in their lactone forms are hydrophobic HMG–CoA reductase inhibitors, and they do not show tissue-selectivity (3,5,6). Lovastatin and simvastatin were taken up by nonhepatic tissue-derived cells, such as fibroblasts, spleen cells as well as hepatocytes, and inhibited sterol synthesis in these cells (3,5). These drugs also inhibited sterol synthesis both in the liver and nonhepatic tissues when orally administered to mice or rats (3,5,6).

These earlier studies (3,5,6), however, were *in vitro* ones or acute ones in which the inhibitors were administered only once. There have been no reports documenting tissue-selectivity of HMG–CoA reductase inhibitors in intact animals after repeated oral administrations, although hypolipidemic drugs are typically used on a long-term basis.

In the present study, we examined whether the liver-selectivity observed in single oral administration experiments in mice is also apparent in long-term experiments, and whether the cellular distribution profiles of pravastatin observed in single oral administration experiments (6) are preserved after repeated oral administrations.

MATERIALS AND METHODS

Materials and animals. [1-^{14}C]Sodium acetate (1.977 GBq/mmol) was obtained from New England Nuclear Co. (Amersham International, Amersham, United Kingdom). Pravastatin (acid form), lovastatin (lactone form), and simvastatin (lactone form) were prepared as previously described (6). Pravastatin was dissolved in 1% Tween 80 aqueous solution (NACALAI TESQUE, Inc., Kyoto, Japan). Lovastatin and simvastatin were suspended in the same solution. Male C57BL/6 mice, eight weeks old (Charles River, Kanagawa, Japan) were used for the experiment.

Determination of sterol synthesis. Sterol synthesis in different tissues of mice *in vivo* was examined by measuring

*To whom correspondence should be addressed at Pharmacology and Molecular Biology Research Laboratories, Sankyo, Co., Ltd., Hiromachi 1-2-58, Shinagawa-ku, Tokyo 140 Japan.

Abbreviation: HMG–CoA; 3-hydroxy-3-methylglutaryl CoA.

Copyright © 1995 by AOCS Press

incorporation of [^{14}C]acetate into digitonin-precipitable [^{14}C]sterols as described previously (5). [^{14}C]Acetate (20 μCi/mouse) was intraperitoneally injected into mice two hours after the last drug administration. One hour later, tissues were excised for measurement of sterol synthesis. To establish a dose-response relationship of inhibition of *de novo* sterol synthesis in the liver, each drug was orally administered once to mice in various doses from 0.03–30 mg/kg, and sterol synthesis in the liver was measured as described above.

Tissue selectivity of HMG–CoA reductase inhibitors after a single oral administration to mice. In order to confirm our previous results (5), we orally administered HMG–CoA reductase inhibitors once to mice at 20 mg/kg, and sterol synthesis inhibition in different tissues of mice was measured as described above. Tissues from four mice were pooled for measurement of sterol synthesis. Sterol synthesis in lens was measured *in vitro* as described previously (5).

Tissue selectivity of HMG–CoA reductase inhibitors after repeated oral administrations to mice. Each drug was orally administered to mice at doses of 5 and 10 mg/kg for 11 consecutive days. Sterol synthesis inhibition in different tissues of mice were measured as described above. Sterol synthesis in lens was measured *in vitro* as described previously (5). Tissues from four mice were pooled for measurement of sterol synthesis.

Determination of sterol synthesis in tissue slices. Pravastatin, lovastatin, and simvastatin were orally administered to mice at 10 mg/kg for 11 consecutive days. Two hours after the last dosing, mice were killed by decapitation, and the liver, spleen, and testis were excised and sliced. Tissues from five mice were pooled for measurement of sterol synthesis. Sterol synthesis in the tissue slices was monitored for two hours using the incorporation of radioactivity from 1 mM [^{14}C]acetate into digitonin-precipitable sterols as described previously (6). The measurement of radioactivity was carried out in duplicate.

Determination of active drug concentration in tissues. After tissue slices were prepared for measurement of sterol synthesis, the active drug concentrations in the remainder of the tissues were determined based on our previous report (6). In brief, the active drugs were extracted from the tissue homogenate with methanol and assayed for inhibition of rat liver HMG–CoA reductase (6). The bioactivity was related to concentrations of the original drug by comparison with concentration-inhibition curves constructed beforehand with known concentrations of the inhibitors.

Determination of serum total cholesterol. Blood samples collected when the mice were sacrificed were left for 30 min at room temperature, and serum was obtained by centrifugation at 2,150 × *g* for 10 min at 4°C. The levels of serum total cholesterol were determined enzymatically using Hitachi type 736 automatic analyzer (Tokyo, Japan).

RESULTS

Inhibition of sterol synthesis in the liver. We examined dose-response relationship of inhibition of *de novo* sterol synthesis in the liver three hours after a single oral administration of HMG–CoA reductase inhibitors, and determined ED_{50} values, drug doses required for 50% inhibition of sterol synthesis in the liver: They were (mean ± SEM, n = 3) 0.6 ± 0.01, 0.56 ± 0.09, and 0.74 ± 0.10 mg/kg for pravastatin, lovastatin, and simvastatin, respectively.

Typical dose-response curves in sterol synthesis inhibition in the liver after a single oral dose of each drug are shown in Figure 1, where 20 mg/kg is a minimum dose at which each drug equally exhibits almost maximal inhibition (more than 80%) of sterol synthesis in the liver. In single oral administration experiments, we compared tissue-selectivity of these three drugs at 20 mg/kg in inhibiting sterol synthesis in different tissues of mice. On the other hand, sterol synthesis inhibition in the liver was less than 80% at doses of 5 and 10 mg/kg. In the long-term experiments, we chose these doses, lower than that in single oral administration experiments, so that chronic effects were not masked by the acute effect of the last dose of drugs.

Tissue selectivity of HMG–CoA reductase inhibitors after a single oral administration to mice. Administration of a single oral dose of pravastatin at 20 mg/kg inhibited sterol synthesis in the liver three hours later by more than 80% (Table 1). This agent, however, inhibited sterol synthesis at this time point by less than 20% in the kidney, spleen, heart, cerebrum, diaphragm, lens, and in the steroid hormone-producing tissues, such as the adrenal and testis (Table 1). On the other hand, lovastatin and simvastatin inhibited sterol synthesis significantly in the nonhepatic tissues, as well as in the liver. These results agreed well with our previous observations (5) that pravastatin, but not lovastatin and simvastatin, was liver-selective in inhibiting sterol synthesis in mice after a single oral dose.

Tissue selectivity of HMG–CoA reductase inhibitors after repeated oral administrations to mice. Inhibition of sterol

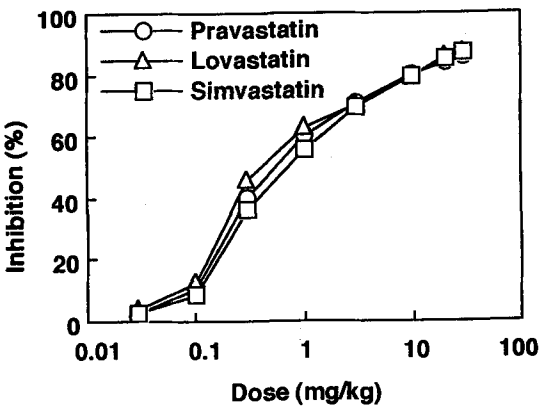


FIG. 1. Dose response curves of inhibition of *de novo* sterol synthesis in the liver. Each drug was orally administered to mice at 0.03–30 mg/kg. Two hours later, [^{14}C]acetate was intraperitoneally injected into mice. One hour after [^{14}C]acetate injection, mice were killed by decapitation, the liver was excised, and sterol synthesis was measured as described in the text. Each point represents the mean from four mice.

TABLE 1
Inhibition of *de novo* Sterol Synthesis in Different Tissues After a Single Oral Dose of Pravastatin, Lovastatin, and Simvastatin in Mice[a]

	(dpm/100 mg tissue)			
	Control	Pravastatin	Lovastatin	Simvastatin
Liver	1278 ± 192	242 ± 38[c] (81.1)	174 ± 31[c] (86.4)	179 ± 33[c] (86.0)
Adrenal	308 ± 30	274 ± 24 (11.0)	223 ± 22[b] (27.6)	173 ± 18[c] (43.8)
Kidney	409 ± 29	328 ± 24[b] (19.8)	187 ± 12[c] (54.2)	225 ± 25[c] (44.9)
Spleen	696 ± 35	617 ± 42 (11.4)	339 ± 12[c] (51.3)	353 ± 18[c] (49.3)
Testis	1055 ± 198	1018 ± 320 (3.5)	377 ± 56[b] (64.3)	261 ± 48[c] (75.3)
Heart	253 ± 23	228 ± 27 (9.9)	182 ± 15[b] (28.1)	185 ± 14[b] (26.9)
Cerebrum	161 ± 8	147 ± 9 (8.7)	135 ± 6[b] (16.1)	115 ± 8[c] (28.3)
Diaphragm	49 ± 8	48 ± 4 (2.0)	22 ± 3[c] (55.1)	23 ± 3[c] (52.0)
Lens	287 ± 32	265 ± 21 (7.7)	183 ± 12[c] (36.2)	188 ± 13[c] (34.5)

[a]Each drug was orally administered to mice at a dose of 20 mg/kg. Two hours later, [^{14}C]acetate was intraperitoneally injected into mice. One hour after [^{14}C]acetate injection, mice were killed by decapitation, and tissues were excised. Tissues from four mice were pooled, and sterol synthesis was measured as described in the text. Values are mean ± SEM (n = 5). Inhibition as percentage of control is shown in parentheses. [b]$P < 0.05$. [c]$P < 0.01$ by Duncan's test.

synthesis in different tissues of mice after repeated oral administrations of HMG–CoA reductase inhibitors at 5 and 10 mg/kg for 11 consecutive days is summarized in Table 2. All three drugs inhibited sterol synthesis by more than 60% in the liver. On the other hand, pravastatin did not inhibit sterol synthesis in nonhepatic tissues even at 10 mg/kg, whereas lovastatin and simvastatin inhibited sterol synthesis by more than 20% at 5 mg/kg and more than 30% at 10 mg/kg in most of the nonhepatic tissues.

Active drug concentrations and inhibition of sterol synthesis in the liver, spleen, and testis. The active drug concentrations and inhibition of sterol synthesis were measured in the liver, spleen, and testis that were excised two hours after the last oral administrations of pravastatin, lovastatin, and simvastatin to mice at a dose of 10 mg/kg for 11 consecutive days. All three drugs were present in higher concentrations in the liver than in the spleen and testis (Table 3). The active drug concentrations of all three drugs were almost identical in the liver (Table 3), where all three drugs inhibited sterol synthesis to a similar degree (Table 4) as they did in the *in vivo* study (Table 2). The active drug concentrations of pravastatin were also comparable to those of lovastatin and simvastatin in the spleen and testis (Table 3), where lovastatin and simvastatin inhibited sterol synthesis, but pravastatin did not (Table 4). These data suggest that pravastatin is liver-selective regarding sterol synthesis inhibition.

Body weight, serum cholesterol, and transaminase levels. Serum total cholesterol levels were not changed with an 11-

TABLE 2
Inhibition of *de novo* Sterol Synthesis in Different Tissues After Repeated Oral Administrations of Pravastatin, Lovastatin, and Simvastatin for Eleven Consecutive Days in Mice[a]

	(dpm/100 mg tissue)						
		Pravastatin		Lovastatin		Simvastatin	
	Control	5 mg/kg	10 mg/kg	5 mg/kg	10 mg/kg	5 mg/kg	10 mg/kg
Liver	1965 ± 265	585 ± 43[c] (70.2)	501 ± 25[c] (74.5)	607 ± 75[c] (69.1)	483 ± 62[c] (75.4)	730 ± 78[c] (62.9)	484 ± 47[c] (75.4)
Adrenal	291 ± 8	310 ± 45 (−6.6)	285 ± 50 (2.1)	204 ± 4 (30.0)	189 ± 14[b] (35.0)	200 ± 8 (31.2)	175 ± 18[b] (39.9)
Kidney	236 ± 15	245 ± 57 (−3.7)	220 ± 8 (6.7)	166 ± 7 (29.9)	163 ± 15 (31.0)	170 ± 10 (28.1)	140 ± 17[b] (40.7)
Spleen	298 ± 18	272 ± 35 (8.7)	268 ± 32 (10.0)	218 ± 14[b] (27.0)	188 ± 13[c] (36.8)	193 ± 12[c] (35.1)	165 ± 16[c] (44.7)
Testis	1044 ± 63	1209 ± 384 (−15.8)	1014 ± 76 (2.9)	672 ± 72 (35.7)	463 ± 70[b] (55.7)	712 ± 96 (31.8)	607 ± 67 (41.9)
Heart	294 ± 26	283 ± 27 (3.8)	267 ± 50 (9.2)	231 ± 23 (21.3)	218 ± 1 (25.8)	221 ± 10 (24.7)	206 ± 15[b] (30.0)
Cerebrum	166 ± 6	169 ± 9 (−1.8)	163 ± 10 (1.5)	131 ± 4[b] (21.0)	112 ± 16[c] (32.6)	141 ± 8 (15.1)	122 ± 9[c] (26.4)
Diaphragm	72 ± 10	71 ± 17 (1.4)	66 ± 7 (8.4)	51 ± 10 (29.0)	48 ± 12 (33.7)	54 ± 5 (24.4)	44 ± 5 (38.3)
Lens	289 ± 72	270 ± 32 (6.7)	282 ± 51 (2.5)	254 ± 22 (12.0)	205 ± 37 (28.9)	208 ± 25 (28.0)	196 ± 26 (32.3)

[a]Each drug was orally administered to mice at doses of 5 and 10 mg/kg for 11 consecutive days. Two hours after the last dosing, [^{14}C]acetate was intraperitoneally injected, and one hour later, tissues were excised. Tissues from five mice were pooled, and sterol synthesis was measured as described in the text. Values are mean ± SEM (n = 4). Inhibition as percentage of control is shown in parentheses. [b]$P < 0.05$. [c]$P < 0.01$ by Duncan's test.

TABLE 3
The Active Drug Concentrations of Pravastatin, Lovastatin, and Simvastatin in the Liver, Spleen, and Testis of Mice[a]

	Pravastatin	Lovastatin	Simvastatin
Liver (ng/g tissue)	488.0 ± 530	539.0 ± 150	394.0 ± 450
Spleen (ng/g tissue)	48.9 ± 3.3	49.8 ± 4.2	49.4 ± 6.2
Testis (ng/g tissue)	46.2 ± 5.1	53.5 ± 4.0	52.5 ± 2.9

[a]After tissue slices were prepared for measurement of sterol synthesis as described in the text, the remainder of the tissues was homogenized, and the drug was extracted with methanol. The active drug concentrations in the methanol extracts were determined by bioassay as described in the text. Assay was carried out in duplicate. Values are mean ± SEM (n = 4).

TABLE 4
Inhibition of *de novo* Sterol Synthesis in the Liver, Spleen, and Testis of Mice[a]

	(dpm/100 mg tissue)			
	Control	Pravastatin	Lovastatin	Simvastatin
Liver	2718 ± 376	773 ± 87[c] (71.6)	635 ± 32[c] (76.7)	594 ± 35[c] (78.1)
Spleen	3809 ± 609	3723 ± 473 (2.3)	1525 ± 422[c] (60.6)	1751 ± 261[b] (54.0)
Testis	7262 ± 490	7442 ± 332 (−2.5)	3803 ± 500[c] (47.6)	2454 ± 406[c] (66.2)

[a]Each drug was orally administered to mice at 10 mg/kg for 11 consecutive days. Two hours after the last drug administration of pravastatin, lovastatin, and simvastatin, mice were killed by decapitation, and the liver, spleen, and testis were excised and sliced. Tissues from five mice were pooled for measurement of sterol synthesis. Sterol synthesis in the slices was measured as described in the text. Assay was carried out in duplicate. Values are mean ± SEM (n = 4). Inhibition as percentage of control is shown in parentheses. [b]$P < 0.05$. [c]$P < 0.01$ by Duncan's test.

TABLE 5
Changes in Serum Total Cholesterol[a]

	Total cholesterol (mg/dL)
Control	86.0 ± 1.8
Pravastatin (10 mg/kg)	85.5 ± 3.0
Lovastatin (10 mg/kg)	84.0 ± 1.4
Simvastatin (10 mg/kg)	82.0 ± 1.0

[a]Each drug was orally administered to mice at 10 mg/kg for 11 consecutive days. Two hours after the last drug administration of pravastatin, lovastatin, and simvastatin, mice were killed by decapitation, blood was collected, and serum was obtained by centrifugation. The levels of serum total cholesterol were determined enzymatically as described in the text. Values are mean ± SEM (n = 4).

day treatment of HMG–CoA reductase inhibitors (Table 5). There were no differences in body weight gain or serum transaminase levels between the control and drug treatment groups after an 11-day treatment with HMG–CoA reductase inhibitors (data not shown).

DISCUSSION

In the present study, we examined if the hepatic selectivity of pravastatin for HMG–CoA reductase inhibition observed in single oral administration experiments *in vivo* was maintained in long-term experiments. We first confirmed the previous results (3,5,6) that after a single oral administration, pravastatin showed greater liver-selectivity in inhibiting sterol synthesis than lovastatin and simvastatin (Table 1). We then examined tissue-selectivity after repeated oral administration of HMG–CoA reductase inhibitors, and we concluded that the difference in liver-selectivity among these three inhibitors observed in a single-dose study was preserved after repeated oral administrations (Tables 2 and 4).

In long-term experiments, the doses used were 5 and 10 mg/kg. We chose these two doses for the following reasons. First, we chose doses which produced less than 80% inhibition of sterol synthesis in the liver (Fig. 1) to minimize the likelihood that chronic effects would be masked by the acute effect of the last dose of drug. We also chose doses at which drugs similarly reduce serum cholesterol. None of these HMG–CoA reductase inhibitors, however, decreased serum cholesterol in our mice (Table 5), and it is known (8) that HMG–CoA reductase inhibitors do not reduce serum cholesterol in rodents. Since inhibition of HMG–CoA reductase in the liver is thought to cause the reduction of serum cholesterol (9–11), we chose doses producing almost the same inhibition of sterol synthesis in the liver instead. In experimental animals, the doses at which HMG–CoA reductase inhibitors decrease serum cholesterol are more than 3 mg/kg when drugs are administered once a day for more than seven days (Koga, T., unpublished data): in dogs, more than 3 mg/kg; in rabbits and common marmosets, more than 5 mg/kg; in cynomolgus monkeys, more than 20 mg/kg. We therefore chose doses more than 3 mg/kg. Since the efficacy of simvastatin was reported to be nearly two times greater than that of pravastatin or lovastatin, we selected two doses, 5 and 10 mg/kg.

Because these reasons, we administered 5 or 10 mg/kg HMG–CoA reductase inhibitors in long-term experiments. Although these doses were higher than those administered to humans (approximately 0.2–0.6 mg/kg), we think that they do not appear to have been toxic to mice because there were no changes of body weight gain and the levels of serum transaminases between the control and drug treatment groups (data not shown). Since these doses were less than those in single oral administration experiments, the inhibition of sterol synthesis in the liver after repeated administrations was somewhat less than that after a single oral administration. Nevertheless, the liver selective inhibition of sterol synthesis with pravastatin and the lack of such selectivity with the other two inhibitors were apparent (Tables 2 and 4).

Conflicting results have been reported with regard to tissue selectivity of HMG–CoA reductase inhibitors (3,5–7,12). Based on inhibition of sterol synthesis in cells *in vitro* (3,5–7), in rat tissue slices (3,6), and in mice *in vivo* (5), we have reported that pravastatin is more liver-selective than lovastatin or simvastatin. These results were confirmed in the present study (Tables 1, 2, and 4). On the other hand, based on the distribution of drugs in the liver relative to nonhepatic tissues, Germershausen *et al.* (12) have concluded that lovastatin and

simvastatin are more liver-selective than pravastatin. In our recent study (6), we resolved this discrepancy. Namely, because pravastatin is distributed in the intracellular space in the liver and remained in the extracellular space in nonhepatic tissues after oral administration to rats, this drug inhibited sterol synthesis more strongly in the liver than in other tissues.

In order to examine whether the cellular distribution profiles of pravastatin in different tissues were maintained after repeated oral dosings, we administered pravastatin, lovastatin, and simvastatin to mice at 10 mg/kg for 11 consecutive days and determined the relationship between active drug concentrations and sterol synthesis inhibition in the liver, spleen, and testis. The levels of active drug concentrations of three HMG–CoA reductase inhibitors were almost comparable in each tissue, and those in the liver were higher than those in the spleen and testis, as we have previously reported in rats (6). On the other hand, pravastatin was liver-selective in inhibiting sterol synthesis, but lovastatin and simvastatin were not. Together with the results of our previous report (6), these results suggest that pravastatin permeated the cell membrane in the liver, but not in nonhepatic tissues, whereas lovastatin and simvastatin penetrated inside the cells in all these tissues. We conclude that the distribution of pravastatin inside hepatocytes and outside the cells in nonhepatic tissues suggested by single dose studies may also be present after repeated oral administrations.

Lovastatin and simvastatin, but not pravastatin, inhibited sterol synthesis in the diaphragm, lens, and cerebrum after a single or repeated oral administrations. Mosley *et al.* (13) also demonstrated that lovastatin, but not pravastatin, inhibited sterol synthesis in rat lens after a single oral dose. Myopathy (14–16) and insomnia (17–19) have been reported for lovastatin and simvastatin, but have not yet been reported for pravastatin. Significant inhibition of sterol synthesis in nonhepatic tissues by these two inhibitors, but not by pravastatin, may have clinical relevance for a number of effects.

ACKNOWLEDGMENTS

We are grateful to Tadashi Koieyama, Naomi Kasanuki, and Kayoko Arai for their skillful assistance in the experiments.

REFERENCES

1. Serizawa, N., Nakagawa, K., Hamano, K., Tsujita, Y., Terahara, A., and Kuwano, H. (1983) Microbial Hydroxylation of MI-236B (compactin) and Monacolin K (MB-530B), *J. Antibiot. 36*, 604–607.
2. Serizawa, N., Serizawa, S., Nakagawa, K., Furuya, K., Okazaki, T., and Terahara, A. (1983) Microbial Hydroxylation of ML-236B (compactin). Studies on Microorganisms Capable of 3β-Hydroxylation of ML-236B, *J. Antibiot. 36*, 887–891.
3. Tsujita, Y., Kuroda, M., Shimada, Y., Tanzawa, K., Arai, M., Kaneko, I., Tanaka, M., Masuda, H., Tarumi, C., Watanabe, Y., and Fujii, S. (1986) CS-514, a Competitive Inhibitor of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase-Tissue Selective Inhibitor of Sterol Synthesis and Hypolipidemic Effect on Various Animal Species, *Biochim. Biophys. Acta 877*, 50–60.
4. Rodwell, V.W., Norstrom, J.L., and Mitshelen, J.J. (1976) Regulation of HMG-CoA Reductase, *Adv. Lipid Res. 14*, 1–74.
5. Koga, T., Shimada, Y., Kuroda, M., Tsujita, Y., Hasegawa, K., and Yamazaki, M. (1990) Tissue-Selective Inhibition of Cholesterol Synthesis *in vivo* by Pravastatin Sodium, a 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor, *Biochim. Biophys. Acta 1045*, 115–120.
6. Koga, T., Fukuda, K., Shimada, Y., Fukami, M., Koike, H., and Tsujita, Y. (1992) Tissue Selectivity of Pravastatin Sodium, Lovastatin and Simvastatin, *Eur. J. Biochem. 209*, 315–319.
7. Komai, T., Shigehara, E., Tokui, T., Koga, T., Ishigami, M., Kuroiwa, C., and Horiuchi, S. (1992) Carrier-Mediated Uptake of Pravastatin by Rat Hepatocytes in Primary Culture, *Biochem. Pharm. 43*, 667–670.
8. Fujioka, T., Nara, F., Tsujita, Y., Fukushige, J., Fukami, M., and Kuroda, M. (1994) The Mechanism of Lack of Hypocholesterolemic Effects of Pravastatin Sodium, a 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor, in Rats, *Biochim. Biophys. Acta 1254*, 7–12.
9. Reihner, E., Rudling, M., Stahlberg, D., Berglund, L., Ewerth, S., Bjorkhem, I., Einarsson, K., and Angelin, B. (1990) Influence of Pravastatin, a Specific Inhibitor of HMG-CoA Reductase, on Hepatic Metabolism of Cholesterol, *N. Engl. J. Med. 323*, 224–228.
10. Ma., P.T., Gil, G., Sudhof, T.T., Bilheimer, D.W., Goldstein, J.L., and Brown, M.S. (1986) Mevinolin, an Inhibitor of Cholesterol Synthesis, Induces mRNA for Low Density Lipoprotein Receptor in Livers of Hamsters and Rabbits, *Proc. Natl. Acad. Sci. USA 83*, 8370–8374.
11. Kume, N., Kita, T., Mikami, A., Yokode, M., Ishii, K., Nagano, Y., and Kawai, C. (1989) Induction of mRNA for Low-Density Lipoprotein Receptors in Heterozygous Watanabe Heritable Hyperlipidemic Rabbits Treated with CS-514 (Pravastatin) and Cholestyramine, *Circulation 79*, 1084–1090.
12. Germershausen, J.I., Hunt. V.M., Bostedor, R.G., Bailey, P.J., Karkas, J.D., and Alberts, A.W. (1989) Tissue Selectivity of the Cholesterol-lowering Agents Lovastatin, Simvastatin and Pravastatin in Rats *in vivo*, *Biochem. Biophys. Res. Commun. 158*, 667–675.
13. Mosley, S.T., Kalinowski, S.S., Schafer, B.L., and Tanaka, R.D. (1989) Tissue-Selective Acute Effects of Inhibitors of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase on Cholesterol Biosynthesis in Lens, *J. Lipid Res. 30*, 1411–1420.
14. Tobert, J.A. (1988) Efficacy and Long-Term Adverse Effect Pattern of Lovastatin, *Am. J. Cardiol. 62*, 128J–134J.
15. Norman, D.J., Illingworth, D.R., Munson, J., and Hosenpud, J. (1988) Myolysis and Acute Renal Failure in a Heart-Transplantation Recipient Receiving Lovastatin, *N. Engl. J. Med. 318*, 46–47.
16. East, C., Alivizatos, P.A., Grundy, S.M., Jones, P.H., and Farmer, J.A. (1988) Rhabdomyolysis in Patients Receiving Lovastatin after Cardiac Transplantation, *N. Engl. J. Med. 318*, 47–48.
17. Tobert, J.A., Shear, C.L., Chremus, A.N., and Mantell, G.E. (1990) Clinical Experience with Lovastatin, *Am. J. Cardiol. 65*, 23F–26F.
18. Barth, J.D., Kruisbrink, O.A., and Van Dijk, A.L. (1990) Inhibitors of Hydroxyglutaryl Coenzyme A Reductase for Treating Hypercholesterolemia, *Br. Med. J. 301*, 669.
19. Vgontzas, A.N., Kales, A., Bixler, E.O., Manfredi, R.L., and Tyson, K.L. (1991) Effects of Lovastatin and Simvastatin on Sleep Efficiency and Sleep Stages, *Clin. Pharmacol. Ther. 50*, 730–737.

[Received January 14, 1994, and in final revised form June 16, 1995; Revision accepted June 19, 1995]

Synthesis of Linoleate and α-Linolenate by Chain Elongation in the Rat

Stephen C. Cunnane[a,*], Mary Ann Ryan[a], Kerr S. Craig[b], Steven Brookes[b], Berthold Koletzko[c], Hans Demmelmair[c], Janet Singer[d] and David J. Kyle[d]

[a]Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto M5S 1A8, Canada, [b]Europa Scientific, Crewe, Cheshire, United Kingdom, [c]Kinderpoliklinik, University of Munich, Munich, Germany and [d]Martek Biosciences Corporation, Columbia, Maryland

ABSTRACT: The objective was to determine whether rats could synthesize longer chain polyunsaturates from hexadecadienoate (16:2n-6) and hexadecatrienoate (16:3n-3). Rats were gavaged with uniformly ^{13}C-labelled hexadecadienoate or hexadecatrienoate, euthanized 24 h later, and total lipids were extracted from liver and carcass. Gas chromatography/combustion/isotope ratio mass spectrometry was used to measure ^{13}C levels in individual liver, carcass, and whole body fatty acids. ^{13}C Enrichment was present in desaturated and chain-elongated polyunsaturates, including linoleate, arachidonate, α-linolenate, and docosahexaenoate at 12–13% of the dose of tracer given. ^{13}C Enrichment from hexadecatrienoate was highest in carcass and liver α-linolenate, representing 3.5 and 17.9% of the total α-linolenate pool, respectively. For linoleate, arachidonate, or docosahexaenoate, the contribution of ^{13}C did not exceed 0.2% of the total body pool. Green leafy vegetables common in the human diet were shown to contain up to 1.2% of total fatty acids as hexadecadienoate and 11.6% as hexadecatrienoate. Hence, humans consuming green vegetables probably synthesize a small proportion of their total body content of linoleate and α-linolenate.

Lipids 30, 781–783 (1995).

Linoleate (18:2n-6) and α-linolenate (18:3n-3) are widely accepted as being essential nutrients in the diets of mammals. Although plants and mammals can insert methylene-interrupted double bonds at the C_{9-10} position to synthesize oleate (18:1n-9) from stearate (18:0), only plants can desaturate 18:1n-9 at the C_{12-13} and C_{15-16} positions to form 18:2n-6 and 18:3n-3, respectively. Once the unsaturated bond is at the C_{9-10} and C_{12-13} positions, mammals can desaturate and chain elongate 18:2n-6 and 18:3n-3 to longer chain polyunsaturate fatty acids (PUFA).

Thirty years ago it was shown that 18:2n-6 and n-6 longer chain PUFA could be synthesized in rats from shorter chain n-6 PUFA, including tetradecadienoate (14:2n-6) and hexadecadienoate (16:2n-6), but not from the 10 or 12 carbon analogues (1). Hexadecatrienoate (16:3n-3) also can be converted to n-3 long-chain PUFA (2). These important observations suggest that 18:2n-6 and 18:3n-3 may not be essential in the mammalian diet if the 14- or 16-carbon precursors are consumed, but they have not, to our knowledge, been confirmed. However, several species of edible plants and marine algae contain 16:2n-6 and 16:3n-3, so it is possible that humans, especially vegetarians, can use dietary 16:2n-6 and 16:3n-3 to synthesize some 18:2n-6 and 18:3n-3.

*To whom correspondence should be addressed.

Abbreviations: GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; PUFA, polyunsaturate fatty acids.

METHODS

The marine unicellular microalgae—*Chlorella vulgaris*—was grown in a closed photobioreactor system in which $^{13}CO_2$ was the sole exogenous source of carbon. Over a period of 21 d, the microalgae uniformly incorporated ^{13}C into the entire biomass at levels exceeding 98%. The total fatty acids (composition in Table 1) of the uniformly (U)-^{13}C-labelled microalgae then were extracted and separated to purify the 16:2n-6 and 16:3n-3 by low temperature crystallization, urea adduction, and preparative high-performance liquid chromatography. These procedures yielded [U-^{13}C]-16:2n-6 and [U-^{13}C]-16:3n-3 at 96% purity. Twenty mg of [U-^{13}C]-16:2n-6 or 16:3n-3 methyl ester was suspended in 200 μL olive oil and gavaged into 200 g Sprague-Dawley rats ($n = 4$ per group; Charles River Canada, St. Constant, Quebec,

TABLE 1
Percent Composition of 16 and 18 Carbon Polyunsaturates in *Chlorella vulgaris* and Some Common Edible Green Vegetables

	16:2n-6	18:2n-6	16:3n-3	18:3n-3
C. vulgaris	8.9	28.1	7.4	12.5
String beans	0.2	22.0	ND	25.3
Green pepper	0.3	22.7	0.2	15.1
Celery	0.2	19.0	0.5	4.0
Green onion	ND	24.6	ND	21.2
Broccoli buds	1.0	14.4	1.3	33.9
Broccoli leaves	1.2	9.2	11.6	45.3
Spinach	ND	9.9	4.9	38.8
Spinach[a]	1.9	11.7	14.6	53.9

[a]From Reference 4. ND, not detected (<0.05%).

Copyright © 1995 by AOCS Press

Canada). The rats were euthanized 24 h later under a CO_2 atmosphere, and the livers were removed for analysis. The remaining carcass was analyzed so that whole body fatty acid and ^{13}C content could be determined.

Liver and carcass total lipids were extracted into chloroform/methanol (2:1), saponified, and weighed. The fatty acids were methylated using 14% boron–trifluoride in methanol. Fatty acid methyl esters were separated by capillary gas chromatography (GC), and the liver and carcass fatty acid profiles were determined relative to the retention times of standard reference mixtures. ^{13}C Enrichment in the fatty acid methyl esters was determined by GC/combustion/isotope ratio mass spectrometry (GC/C/IRMS) (3). The ^{13}C enrichment in 18:2n-6 isotopomers of an aliquot of the liver fatty acid mixture was determined using a mass selective GC/MS equipped with an electron impact detector (Hewlett-Packard, Palo Alto, CA).

To determine possible sources of 16:2n-6 and 16:3n-3 in human diets, the fatty acid profiles of the lipids of several common edible green vegetables were determined by capillary GC and GC/MS following the same lipid extraction procedures as for rat liver lipids.

RESULTS AND DISCUSSION

Twenty-four hours after dosing with [U-^{13}C] 16:2n-6, rat liver total lipids contained ^{13}C in several n-6 PUFA, including 18:2n-6, dihomo-γ-linolenate (20:3n-6), and arachidonate (20:4n-6) in the range of 1.40 to 1.52 atom%. After dosing with [U-^{13}C]-16:3n-3, liver 18:3n-3, eicosapentaenoate (20:5n-3), and docosahexaenoate (22:6n-3) contained ^{13}C ranging from 1.32 to 3.48 atom%. Carcass enrichments were generally lower than those in liver (Fig. 1). Dosing with ^{13}C-16:2n-6 did not statistically affect ^{13}C levels in n-3 PUFA, and *vice versa* for ^{13}C-16:3n-3. No significant ^{13}C enrichment was observed in saturates or monounsaturates in rat liver after either [U-^{13}C]-16:2n-6 or 16:3n-3, indicating that, within the study period (24 h), there was little recycling of ^{13}C into lipids synthesized *de novo* (data not shown).

To determine the percentage of tracer recovered in body lipids and the change in body content of PUFA induced by the tracers, absolute amounts of the main PUFA and their ^{13}C enrichments were calculated from the atom percent data and the quantitative content of total fatty acids in the liver and carcass of both undosed control rats and those given the tracers. The percentage increase in the carcass pool of PUFA after dosing with the ^{13}C-16:2n-6 or 16:3n-3 ranged from below detection limits for 22:6n-3 to 3.45% for 18:3n-3; in liver these values ranged from 0.13% for 16:2n-6 itself to 17.9% for 18:3n-3 (Tables 2 and 3). Liver and carcass data were combined to give whole body values for both the increase in PUFA and the retention of the tracer. The percent change in whole body content of PUFA induced by the tracers ranged from 0.05% for 22:6n-3 to 3.54% for 18:3n-3; these changes accounted for 12–13% of the tracer given.

The actual amount (mg) of ^{13}C-16:2n-6 incorporated into 18:2n-6 was the same as for ^{13}C-16:3n-3 incorporation into

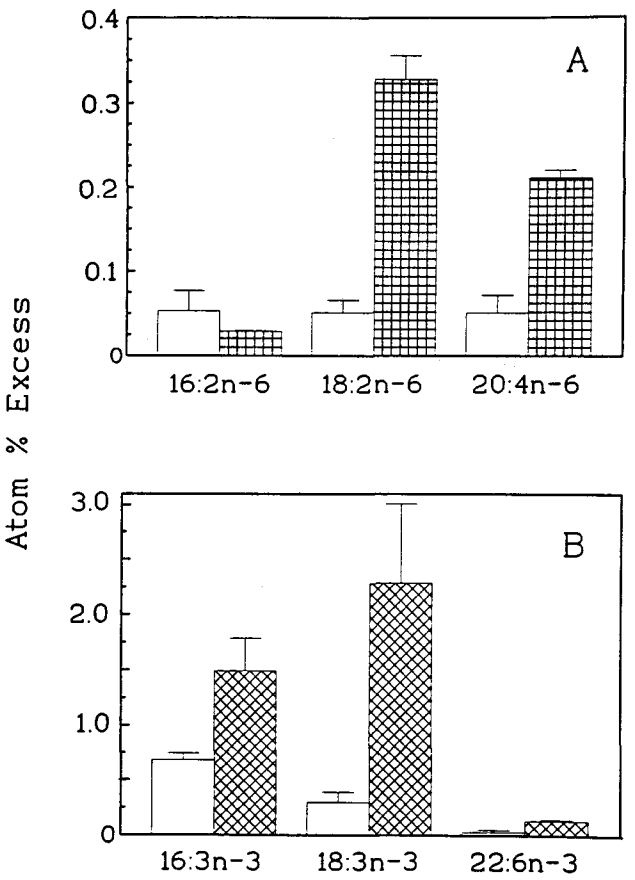


FIG. 1. ^{13}C atom% excess in carcass (open bar) and liver (bars with straight lines, bar with diagonal lines) of rats given an oral dose of 20 mg of [U-^{13}C]-16:2n-6 (A), or [U-^{13}C]-16:3n-3 (B) 24 h earlier.

18:3n-3 (about 2.3 mg each). However, because the whole body pool of 18:2n-6 was twenty times larger than that of 18:3n-3, the increase in the whole body 18:2n-6 caused by the tracer was only 0.18% compared to 3.54% for 18:3n-3 (Tables 2 and 3). Calculated by GC/MS, the change in 18:2n-6 caused by the mass + 16 isotopomer of 18:2n-6, which would have been derived *via* synthesis from [U-^{13}C] 16:2n-6, was approximately 0.4% of the total 18:2n-6 isotopomers in rat liver (data not shown). This value broadly agrees with the calculation of absolute change in 18:2n-6 determined by GC and GC/C/IRMS. The ^{13}C enrichment data suggest that, given an equal dose of 16:2n-6 or 16:3n-3 and an approximately equal body content of 16:2n-6 and 16:3n-3, equal amounts (mg) of ^{13}C-labelled 18:3n-3 and 18:2n-6 are accumulated in rat liver and carcass, but the relative effect on the 18:2n-6 pool is much less due to a 20-fold dilution (Tables 2 and 3).

These results confirm earlier reports that rats are capable of chain-elongating 16:2n-6 to 18:2n-6 and n-6 longer chain PUFA (1), and chain-elongating 16:3n-3 to 18:3n-3 and n-3 longer chain PUFA (2). To our knowledge, the present study reports the first direct comparison of the two 16 carbon precursors in rats already consuming an adequate amount of 18:2n-6 and 18:3n-3; some of the previous work on this topic has involved rats deficient in 18:2n-6 (1), which enhances de-

TABLE 2
Change in Rat Carcass, Liver, and Whole Body Content of n-6 Polyunsaturates and ^{13}C 24 h After Giving an Oral Dose of [U-^{13}C] 16:2n-6

	16:2n-6	18:2n-6	20:4n-6
Carcass[a]			
%	0.25 ± 0.02[b]	19.5 ± 0.6	2.85 ± 0.42
mg	16.0 ± 3.8	1251 ± 326	179 ± 24
mg ^{13}C	0.18 ± 0.05	14.2 ± 3.8	2.02 ± 0.27
Δ mg ^{13}C[c]	+0.03 ± 0.02	+2.21 ± 2.94	+0.20 ± 0.20
% of Pool[d]	0.19	0.18	0.11
Liver[e]			
%	0.13 ± 0.01	15.1 ± 0.7	19.1 ± 1.8
mg	0.16 ± 0.01	18.1 ± 0.8	22.0 ± 2.1
mg ^{13}C	<0.01	0.28 ± 0.19	0.32 ± 0.23
Δ mg ^{13}C	+<0.01	+0.08 ± 0.07	+0.07 ± 0.07
% of Pool	0.13	0.44	0.32
Whole body			
Δ mg ^{13}C	+0.03 ± 0.02	+2.29 ± 2.90	+0.27 ± 0.20
% Dose	0.15	11.45	1.35
% of Pool	0.19	0.18	0.13

[a]6445 mg Total fatty acids/carcass.
[b]Mean ± SD.
[c]Compared to natural abundance values.
[d]Change relative to absolute amount (mg) in carcass, liver, or whole body.
[e]120 mg Total fatty acids/liver.

saturase activity and may therefore increase the conversion of 16:2n-6 to n-6 longer chain PUFA. We also used stable isotope methodology in this study, which is applicable to addressing the same question in humans.

Because the amount of 16:2n-6 and 16:3n-3 in several common edible green plants is up to 1.2 and 11.8%, respectively (Table 1), we infer that humans probably synthesize some of the 18:2n-6 and 18:3n-3 present in the body. The extent that the whole body content of 18:2n-6 or 18:3n-3 is synthesized vs. consumed must vary partly as a function of the ratio of 16:2n-6/18:2n-6 and 16:3n-3/18:3n-3 in the overall diet. In the few green plants analyzed, total 16:2n-6 was highest in broccoli leaves at 13.0% of the 18:2n-6 content. Also in broccoli, 16:3n-3 was 3.8% of the content of 18:3n-3 in the buds and 25.6% of the 18:3n-3 in broccoli leaves (Table 1); 16:3n-3 is also present in spinach (4). Hence, some common edible green vegetables may contribute appreciable amounts of 16:3n-3 to the human diet.

In true vegetarians (vegans), green plants would be an important source of 18:3n-3, but 18:2n-6 is found in a wider range of seeds, pulses, and legumes. If the green plants we analyzed here for 16:2n-6 and 16:3n-3 are representative of those most commonly found in the human diet, and if rats and humans metabolize hexadecadienoate and hexadecatrienoate at a similar rate, about 3–4% of the body's 18:3n-3, but <1% of its 18:2n-6, could be synthesized from their respective 16 carbon precursors. Rats actually appear to synthesize more 22:6n-3/g 18:3n-3 consumed than humans, and the human consumption of 16:3n-3 likely would not exceed 10% of 18:3n-3 intake, even in vegetarians (Table 1). Thus, it seems probable that 18:3n-3 synthesis from 16:3n-3 would account for <1% of the whole body pool, whereas for 18:2n-6, it would probably be <0.1%. From these estimates, there seems to be no doubt that 18:2n-6 and 18:3n-3 remain functionally essential in the human diet even though, extrapolating from the present data obtained in rats, humans can probably synthesize them by chain-elongation.

TABLE 3
Change in Rat Carcass, Liver, and Whole Body Content of n-3 Polyunsaturates and ^{13}C 24 h After Giving an Oral Dose of [U-^{13}C] 16:3n-3

	16:3n-3	18:3n-3	22:6n-3
Carcass[a]			
%	0.52 ± 0.16[b]	1.15 ± 0.27	1.84 ± 0.54
mg	23.7 ± 12.4	64.9 ± 40.8	87.1 ± 17.0
mg ^{13}C	0.43 ± 0.23	2.98 ± 2.38	0.97 ± 0.19
Δ mg ^{13}C[c]	+0.18 ± 0.20	+2.24 ± 2.38	<0.01
% of Pool[d]	0.76	3.45	<0.01
Liver[e]			
%	0.17 ± 0.01	0.33 ± 0.07	11.48 ± 2.04
mg	0.21 ± 0.01	0.39 ± 0.08	13.82 ± 2.44
mg ^{13}C	<0.01	0.01 ± 0.01	0.18 ± 0.15
Δ mg ^{13}C	+<0.01	+0.07 ± 0.08	+0.05 ± 0.06
% of Pool	1.90	17.93	0.36
Whole body			
Δ mg ^{13}C	+0.18 ± 0.16	+2.31 ± 2.18	+0.05 ± 0.06
% of Dose	0.90	11.55	0.25
% of Pool	0.75	3.54	0.05

[a]5250 mg Total fatty acids/carcass.
[b]Mean ± SD.
[c]Compared to natural abundance values.
[d]Change relative to absolute amount (mg) in carcass, liver, or whole body.
[e]120 mg Total fatty acids/liver.

ACKNOWLEDGMENTS

The Natural Sciences and Engineering Research Council of Canada and Bundes Ministerium für Forshung (Germany) provided financial support for this research.

REFERENCES

1. Sprecher, H. (1968) The Total Synthesis and Metabolism of 4-Decaenoate, Dodeca-3,6-Dienoate, Tetradeca-5,8-Dienoate and Hexadeca-7,10-Dienoate in the Fat-Deficient Rat, *Lipids 3*, 14–20.
2. Klenk, E. (1965) The Metabolism of Polyenoic Fatty Acids, *Adv. Lipid Res. 3*, 1–23.
3. Cunnane, S.C., Williams, S.C.R., Bell, J.D., Brookes, S., Craig, K., Iles, R.A., and Crawford, M.A. (1994) Utilization of [U-^{13}C]-Polyunsaturated Fatty Acids in the Synthesis of Long Chain Fatty Acids and Cholesterol Accumulating in the Neonatal Rat Brain, *J. Neurochem. 62*, 2429–2436.
4. Simopoulos, A.P., Norman, H.A., Gillaspy, J.E., and Duke, J.A. (1992) Common Purslane: A Source of Omega-3 Fatty Acids and Antioxidants, *J. Am. Coll. Nutr. 11*, 374–382.

[Received December 5, 1994, and in final revised form June 5, 1995; Revision accepted June 5, 1995]

ERRATUM

In *Lipids,* Vol. 30, no. 5 (May 1995), "Leukotriene B_4 Stimulation of an Early Elevation of Phosphatidic Acid Mass in Human Neutrophils," by Jen-sie Tou and Thao Dola, pp. 373–381, the third line in the legend to Figure 2 should read "pmoles in 5 μL chloroform were added ..." In the legend to Figure 5, an abbreviation was incorrectly spelled out. In the first line of the legend, "phosphatidylethanolamine (PtdEt)" should read "phosphatidylethanol (PtdEt)."

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) Temperature and Diet Effects on Trout, *J. Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) Temperature and Diet Effects on Trout, in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., Temperature and Diet Effects on Trout, *J. Biol. Chem.*, in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

CALENDAR

September 1995

5th International Congress on Platelet-Activating Factor and Related Lipid Mediators, Sept. 12–16, Berlin, Germany. Information: Dr. S. Nigam, Dept. of Gyn., University-Klinikum Steglitz, Hindenburgdamm 30, 12200 Berlin, Germany (Fax: 8155510).

October 1995

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

(continued on next page)

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

American College of Nutrition 36th Annual Meeting, Oct. 13–15, 1995, Omni Shoreham Hotel, Washington, D.C. Information: American College of Nutrition, c/o Hospital for Joint Disease, 301 E. 17th St., New York, NY 10003 (Fax: 212-777-1103).

Lipidforum Seminar: Lipids in Animal Nutrition, Oct. 25–26, 1995, Research Centre Foulum, Tjele (Viborg), Denmark. Information: G. Lambertsen, Lipidforum, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-32 57 24).

November 1995

XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

February 1996

Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

July 1996

European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

July–August 1997

Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Antioxidant Stoichiometry and the Oxidative Fate of Vitamin E in Peroxyl Radical Scavenging Reactions

Daniel C. Liebler* and Jeanne A. Burr

Department of Pharmacology and Toxicology and Southwest Environmental Health Sciences Center, College of Pharmacy, University of Arizona, Tucson, Arizona 85721

ABSTRACT: Oxidation of *R,R,R*-α-tocopherol (vitamin E; TH) by peroxyl radicals generated from the azo initiator azo*bis*(2,4-dimethylvaleronitrile) in acetonitrile, hexane, or in phospholipid liposomes yields 8a-(alkyldioxy)tocopherone adducts, 8a-(hydroxy)tocopherone, and their hydrolysis product α-tocopherolquinone. TH oxidation also yields 4a,5-epoxy- and 7,8-epoxy-8a-(hydroperoxy)tocopherones and their respective hydrolysis products 2,3-epoxy-α-tocopherolquinone and 5,6-epoxy-α-tocopherolquinone. Previous work indicates that the distribution of TH oxidation products varies with reaction environment. We investigated the dependence of antioxidant stoichiometry on TH oxidation product distribution for reactions in hexane, acetonitrile, and in phosphatidylcholine liposomes. Yields of 8a-substituted tocopherones were highest in hexane and lowest in phosphatidylcholine liposomes. In contrast, yields of epoxide products were highest in the liposome system and lowest in hexane. Yields of α-tocopherolquinone were similar in all three systems. Antioxidant stoichiometry, measured by the inhibited autoxidation method, was approximately 2.0 peroxyl radicals trapped per TH consumed in acetonitrile and in liposomes. In hexane, a slightly larger stoichiometric factor of approximately 2.5 was measured. This may, in part, reflect the generation of more reactive alkoxyl radicals in hexane. The reaction environment thus markedly affects the balance between competing TH oxidation pathways but produces comparatively little effect on antioxidant stoichiometry. These results imply that competing reaction pathways contribute similarly to the antioxidant chemistry of TH.

Lipids 30, 789–793 (1995).

α-Tocopherol [vitamin E; 2′*R*,4′*R*,8′*R*-α-tocopherol (*R,R,R*-α-tocopherol) (TH)] is thought to protect tissues by scavenging lipid peroxyl radicals that would otherwise damage biological membranes and other lipid-containing structures (1–3). TH reacts readily with peroxyl radicals to yield a hydroperoxide and the resonance-stabilized α-tocopheroxyl radical (T˙). Further reactions of T˙ yield two groups of nonradical products (4–9). The first consists of 8a-substituted tocopherones, which result either from peroxyl radical addition at C-8a to form 8a-(alkyldioxy)tocopherones (**1**, Scheme 1) (4,6,7,10) or from electron transfer followed by hydrolysis to yield 8a-(hydroxy)tocopherones **2** (9,11,12). Products **1** and **2** hydrolyze and rearrange to form α-tocopherolquinone **3** (13). Both **1** and **2** arise from two-electron oxidations of TH compatible with the previously reported stoichiometry of two peroxyl radicals trapped for each TH oxidized (4,14,15).

The second product group consists of epoxytocopherones **4/5** and their hydrolysis products 2,3-epoxy-α-tocopherolquinone **6** and 5,6-epoxy-α-tocopherolquinone **7** (7,16). The mechanism of TH oxidation to epoxides **4/5** is not known. However, formation of epoxides **4/5** represents a four-electron oxidation of TH and involves the introduction of oxygen as a hydroperoxide moiety at C-8a, possibly *via* an intermediate peroxyl radical (5,7). These considerations prompted us to hypothesize that epoxide formation represented an autoxidation pathway, in which TH is oxidized without consuming peroxyl radicals (7). If so, epoxide formation would reduce the antioxidant stoichiometric factor *n* for TH to values less than two. This is particularly important in view of the considerable variation of epoxide yield with the polarity of the reaction medium. We have reported previously that epoxide yield is highest in polar solvents and in lipid bilayers (16,17). Here we have measured both TH antioxidant stoichiometry and the distribution of TH oxidation products in three different model oxidation systems. The results indicate that TH antioxidant stoichiometry does not vary significantly with epoxide yield and suggests that different TH reaction pathways contribute similarly to radical scavenging by TH.

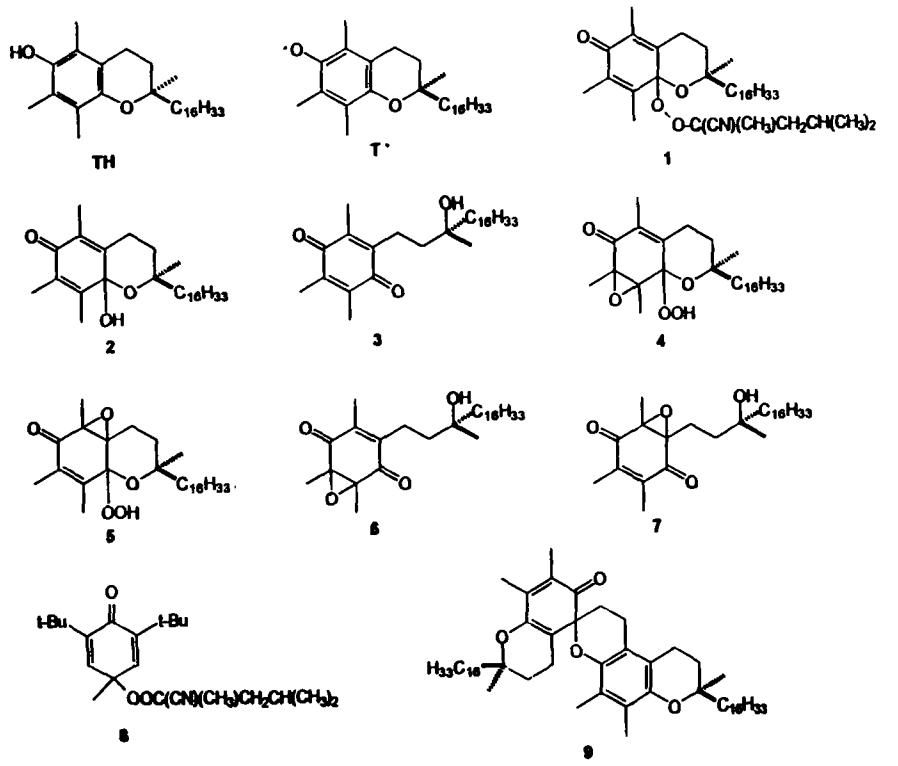
EXPERIMENTAL PROCEDURES

Chemicals and instrumentation. TH (99%) was a gift from Henkel Corporation (La Grange, IL). 2,6-Di-*tert*-butyl-4-methylphenol (BHT) and *N,N′*-diphenyl-1,4-phenylenediamine (DPPD) were purchased from Aldrich (Milwaukee, WI). DPPD was recrystallized twice from ethanol. Azo*bis*-(2,4-dimethylvaleronitrile) (AMVN) was from Polysciences, Inc. (Warrington, PA). Methyl linoleate was from Nu-Chek-Prep (Elysian, MN). L-α-Phosphatidylcholine (from soybean) was from Sigma (St. Louis, MO). [^{14}C]-TH was synthesized

*To whom correspondence should be addressed.

Abbreviations: AMVN, azo*bis*(2,4-dimethylvaleronitrile); BHT, 2,6-di-*tert*-butyl-4-methylphenol; DPPD, *N,N′*-diphenyl-1,4-phenylenediamine; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; TH, 2′*R*,4′*R*,8′*R*-α-tocopherol (*R,R,R*-α-tocopherol); T˙, α-tocopheroxyl radical.

Copyright © 1995 by AOCS Press



SCHEME 1

as described previously (7,18). Gas chromatography/mass spectrometry (GC/MS) analyses were done on a Fisons-VG MD-800 instrument (Fisons Instruments, Beverly, MA) by electron ionization at 70 eV.

Antioxidant stoichiometry measurements. Antioxidant stoichiometric factors *n* were measured by a modification of the method of Boozer *et al.* (19). The accumulation of lipid conjugated dienes, rather than the consumption of oxygen, was used to monitor oxidation of the lipid substrate and its inhibition by antioxidants. For homogeneous solution oxidations, methyl linoleate, antioxidant (BHT or DPPD), and AMVN were dissolved in solvent preheated to 50°C to start the reaction. Reactions in hexane or acetonitrile contained 200 μmol methyl linoleate, 400 nmol antioxidant, and 20 μmol AMVN in a volume of 3 mL. Phosphatidylcholine liposomes contained 50 μmol soybean phosphatidylcholine, 10 μmol AMVN, and 50 nmol antioxidant in a volume of 10 mL. Liposomes were prepared by evaporating stock solutions of all components together under N_2 and then suspending the lipid residue in 50 mM Tris HCl, pH 7.0 preheated to 50°C and then dispersing the mixture in a bath sonicator for 30 s. Incorporation of TH into the liposomes was approximately 100%, as liquid scintillation counting of washes from flasks used to prepare [^{14}C]-TH-containing liposomes (see below) contained only background levels of radioactivity. To monitor substrate autoxidation, 50 μL samples were removed periodically from the reaction mixture, diluted with 1 mL acetonitrile (or 1 mL methanol for liposomal samples), and the absorbance of the sample at 235 nm was measured. Plots of conjugated diene absorbance vs. time were used to determine τ. For each system, 3–6 runs with the antioxidant standard (BHT or DPPD) were done, and an R_i value was calculated. Then 3–6 additional runs were done in which TH was substituted for the antioxidant standard and an *n* value was calculated from Equation 1 (see the Results and Discussion section).

Analysis of [^{14}C]-TH oxidation products. Oxidations contained [^{14}C]-TH (approximately 0.01 μCi) instead of unlabeled TH and all reagent concentrations, and volumes were one-tenth those described above. At the end of the induction period τ for each system, the reaction mixture was prepared for high-performance liquid chromatography (HPLC) analysis as follows. For the acetonitrile system, the entire reaction mixture was injected directly onto the column. For the hexane system, the reaction mixture was evaporated under N_2 and then redissolved in 0.3 mL acetonitrile for HPLC analysis. For the liposome system, one volume of ethanol was added, and the mixture was then extracted three times with two volumes of hexane. The hexane extracts were evaporated under N_2 and then redissolved in 0.3 mL acetonitrile for HPLC analysis. HPLC analyses were done with a Spherisorb ODS-2, 5 μm, 4.6 × 250 mm analytical column (Alltech, Deerfield, IL) eluted with methanol/1N sodium acetate, pH 4.25 (93:7, vol/vol), at a flow rate of 1.5 mL min^{-1}. Fractions (0.8 min) were collected and assayed for radioactivity by liquid scintillation counting. HPLC elution of TH and products **1–7** corresponded to that observed for authentic standards.

Identities of quinone **3** and epoxyquinones **6/7** were further confirmed by GC/MS analysis. Compounds **3** and **6/7**

were converted to trimethylsilyl ethers by treatment with N,O-(*bis*trimethylsilyl)trifluoroacetamide/dimethylformamide (1:1, vol/vol) containing 10% trimethylchlorosilane at room temperature for one hour. Compounds then were analyzed by GC/MS on a DB-5 30 m × 0.25 mm column (J&W Scientific, Folsom, CA) by on-column injection at 150°C. The column was then programmed to 280°C at 15°C min^{-1} and held at that temperature for 10 min. Mass spectra were recorded in the electron ionization mode at 70 eV. Compound **3**: *m/z* 518 ($M^{+\cdot}$, not observed), 430 (44%), 341 (13), 293 (25), 239 (8), 205 (9), 165 (100); compound **6**: *m/z* 534 ($M^{+\cdot}$, not observed), 430 (5%), 341 (41), 309 (100), 267 (61), 209 (14), 165 (15), 137 (19); compound **7**: *m/z* 534 ($M^{+\cdot}$, not observed), 430 (5%), 341 (59), 309 (100), 211 (66), 165 (17), 143 (73).

RESULTS AND DISCUSSION

To assess how changes in TH oxidation product distribution affected the antioxidant stoichiometry for TH, we measured both the antioxidant stoichiometry and the product distribution in hexane, in acetonitrile, and in phospholipid liposomes. Previous studies with the TH model compound 2,2,5,7,8-pentamethylchroman-6-ol indicated that product distributions changed dramatically with increasing polarity of the reaction environment (17). The stoichiometry of radical trapping was measured by the kinetic method originally described by Boozer *et al.* (19) and employed by other investigators to measure the rate constants and stoichiometry for peroxyl radical trapping by TH in solutions (15,20), in sodium dodecyl sulfate micelles (14), and in lipid bilayers (21,22). The stoichiometric factor for peroxyl radical trapping, *n*, is calculated from Equation 1

$$n = R_i\tau/[\mathrm{ArOH}] \quad [1]$$

where R_i is the rate of chain initiation, [ArOH] is the concentration of the antioxidant, and τ is the length of induction period, during which substrate autoxidation is suppressed by the inhibitor. The azo compound AMVN was used to generate peroxyl radicals at a constant rate in each system. The oxidizable substrates used were methyl linoleate (in hexane and acetonitrile) and linoleoyl and linolenoyl acyl chains in the soybean phosphatidylcholine liposomes. Substrate oxidation was monitored by spectrophotometric measurement of conjugated diene accumulation.

To ensure the validity of estimates of R_i obtained with this method, we used two different standard antioxidants, BHT and DPPD. HPLC analyses of reactions in which DPPD was oxidized by AMVN in acetonitrile indicated that *N,N'*-diphenylbenzoquinonimine was the sole detectable product, as was previously reported (19) (data not shown). Likewise, analysis of BHT products formed by oxidation with AMVN in acetonitrile indicated that cyclohexadienone-peroxyl radical adduct **8**, which is analogous to that previously reported from oxidations with azo*bis*(isobutyronitrile) (23), was the predominant product (data not shown). These considerations support the validity of assigning an antioxidant stoichiometric factor *n* of 2 for these antioxidant standards.

BHT and DPPD were used as antioxidant standards to determine R_i values for autoxidations initiated at 50°C by AMVN-derived peroxyl radicals in hexane or acetonitrile solutions containing methyl linoleate as the autoxidation substrate or in soybean phosphatidylcholine liposomes (Table 1). Plots of conjugated diene accumulation vs. time for oxidations of TH in acetonitrile and phosphatidylcholine liposomes are depicted in Figure 1. Sharp breaks in the oxidation curves permitted reproducible estimates of τ (Table 1). The R_i values then were used to estimate stoichiometric factor *n* for TH in each system (Table 1). Values for *n* determined in both acetonitrile and in liposomes were 2.0, within experimental error, which is consistent with previous reports (4,14,15).

The *n* values determined in hexane averaged approximately 2.5 (Table 1), which is slightly greater than would be expected. The reason for the greater *n* value in hexane is not clear. This result may be due to the apparently increased propensity of azo compounds to generate alkoxyl radicals in nonpolar solvents (17,23). Alkoxyl radicals appear to increase the yields of dimer products from TH, and the dimers may

TABLE 1
Antioxidant Kinetic Parameters, Oxidation Product Yields, and *n* Values for Antioxidant Reactions of TH in Hexane, Acetonitrile, and Phosphatidylcholine Liposomes

Reaction medium	Antioxidant standard[a]	τ_{std} (min)	R_i (mM min^{-1})	τ_{TH} (min)	*n*, TH	Product yield (%) 1	3	Epoxides
Hexane	BHT	36.0 ± 0.7 (3)[b]	0.0068 ± 0.0002	47.3 ± 2.1 (3)	2.58 ± 0.11	72 ± 1	4 ± 5	15 ± 1
	DPPD	40.4 ± 6.3 (5)	0.0067 ± 0.0010	47.3 ± 2.1 (3)	2.54 ± 0.11			
Acetonitrile	BHT	32.5 ± 0.5 (3)	0.0082 ± 0.0001	32.5 ± 2.4[c] (4)	2.00 ± 0.15	46 ± 1	10 ± 1	27 ± 1
	DPPD	25.5 ± 3.1 (6)	0.0103 ± 0.0012	26.2 ± 1.3[c] (4)	2.08 ± 0.11			
Liposomes	BHT	45.5 ± 4.5 (5)	0.00022 ± 0.00002	46.0 ± 5.4 (5)	1.88 ± 0.16	41 ± 1	7 ± 0	43 ± 2
	DPPD	37.6 ± 4.6 (5)	0.00027 ± 0.00003	46.0 ± 5.4 (5)	2.28 ± 0.19			

[a]Incubations in hexane and acetonitrile contained 0.133 mM antioxidant, except for incubations with 2,6-di-*tert*-butyl-4-methylphenol (BHT) in hexane, which contained 0.125 mM BHT. Incubations in phosphatidylcholine liposomes contained 0.005 mM antioxidant.
[b]Numbers of determinations are given in parentheses; R_i, rate of chain initiation; τ, length of induction period; *n*, the stoichiometric factor for peroxyl radical trapping.
[c]Separate estimates of lag time τ for 2'*R*,4'*R*,8'*R*-α-tocopherol (*R,R,R*-α-tocopherol) (TH) for oxidations in acetonitrile reflect the use of different lots of methyl linoleate in oxidations standardized with BHT and *N,N'*-diphenyl-1,4-phenylenediamine (DPPD).

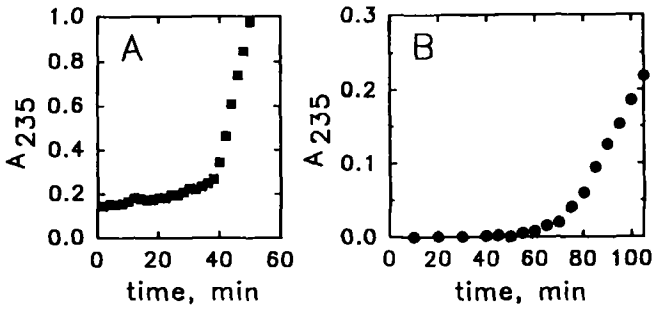


FIG. 1. A) Inhibited autoxidation at 50°C of 66.7 mM methyl linoleate initiated by 6.7 mM azo*bis*(2,4-dimethylvaleronitrile) (AMVN) in acetonitrile containing 0.133 mM 2′*R*,4′*R*,8′*R*-α-tocopherol (*R*,*R*,*R*-α-tocopherol) (TH). B) Inhibited autoxidation at 50°C of phosphatidylcholine liposomes (25 mM in 50 mM Tris HCl, pH 7.0) containing 20 mol% AMVN and 0.1 mol% TH. Autoxidation was monitored in both experiments by measurement of conjugated diene absorbance at 235 nm.

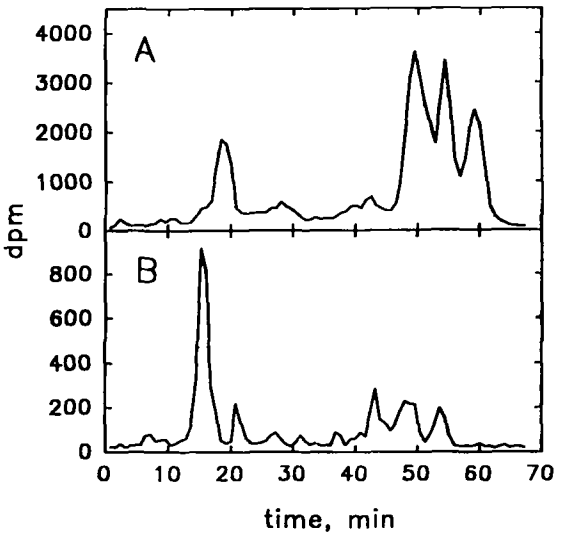


FIG. 2. Reverse-phase high-performance liquid chromatography analyses of products formed from AMVN-initiated oxidation of [^{14}C]-TH in hexane (panel A) and in phosphatidylcholine liposomes (panel B). Epoxides **4**/**5** and their epoxyquinone hydrolysis products **6**/**7** elute between 15–20 min, α-tocopherolquinone **3** elutes at 21 min, and four diastereomers of 8a-(alkyldioxy)tocopherone **1** elute between 40–60 min. See Figure 1 for abbreviations.

themselves trap radicals. However, the yield of dimers in our oxidations in hexane was apparently small (see below). Alkoxyl radicals are considerably more reactive that peroxyl radicals and also may be trapped by other TH oxidation products formed in this system.

Product distribution from TH oxidation was measured in incubations where [^{14}C]-TH was substituted for unlabeled TH. At the time corresponding to the end of the induction period for each system, the run was terminated and residual [^{14}C]-TH and oxidation products were extracted and analyzed by reversed-phase HPLC. Figure 2 depicts HPLC separations of oxidation products formed from [^{14}C]-TH in hexane and in phosphatidylcholine liposomes. Product yields are listed in Table 1. As we had observed previously (16,17), TH product distribution is markedly affected by the reaction medium. In hexane, epoxides are minor products, whereas in liposomes, epoxides account for nearly half of the products. In the acetonitrile and liposome systems, virtually all of the product radioactivity eluted under the chromatographic conditions employed. However, TH spiro dimer **10** was formed during oxidations in hexane, but was not eluted from the HPLC column by the methanol/water mobile phase employed. Parallel analyses with a methanol/ethyl acetate (1:1, vol/vol) mobile phase indicated that the dimer was produced in approximately 5% yield.

These results demonstrate that the antioxidant stoichiometry for TH in peroxyl radical trapping reactions is largely independent of the overall distribution of TH oxidation products. Our earlier speculation (7) that oxidation to epoxides is an autoxidation reaction is probably incorrect. Our present results clearly indicate that increased epoxide formation does not significantly affect antioxidant stoichiometry. Although the mechanism by which the epoxides are formed remains unknown, these data strongly suggest that oxidation of TH to either epoxide **4** or to epoxide **5** results in a net consumption of two peroxyl radicals. Epoxide products **4**–**7**, along with tocopherones **1**–**2** and α-tocopherolquinone **3**, all may serve as biochemical markers for the occurrence of TH antioxidant chemistry in biological membranes. Further investigation of these products as markers for antioxidant function in living tissues are in progress in our laboratory.

ACKNOWLEDGMENTS

This work was supported by USPHS Grants CA47943, CA59585, and ES06694.

REFERENCES

1. Packer, L., and Fuchs, J.L. (eds.) (1993) *Vitamin E in Health and Disease*, Marcel Dekker, Inc., New York.
2. Burton, G.W., and Ingold, K.U. (1986) Vitamin E: Application of the Principles of Physical Organic Chemistry to the Exploration of Its Structure and Function, *Acc. Chem. Res. 19*, 194–201.
3. Chow, C.K. (1991) Vitamin E and Oxidative Stress, *Free Radic. Biol. Med. 11*, 215–232.
4. Winterle, J., Dulin, D., and Mill, T. (1984) Products and Stoichiometry of Reaction of Vitamin E with Alkylperoxy Radicals, *J. Org. Chem. 49*, 491–495.
5. Matsuo, M., Matsumoto, S., Iitaka, Y., and Niki, E. (1989) Radical Scavenging Reactions of Vitamin E and its Model Compound, 2,2,5,7,8-Pentamethylchroman-6-ol, in a Tert-Butylperoxyl Radical Generating System, *J. Am. Chem. Soc. 111*, 7179–7185.
6. Yamauchi, R., Matsui, T., Satake, Y., Kato, K., and Ueno, Y. (1989) Reaction Products of Alpha-Tocopherol with a Free Radical Initiator, 2,2′-Azo*bis*(2,4-dimethylvaleronitrile), *Lipids 24*, 204–209.
7. Liebler, D.C., Baker, P.F., and Kaysen, K.L. (1990) Oxidation of Vitamin E: Evidence for Competing Autoxidation and Peroxyl Radical Trapping Reactions of the Tocopheroxyl Radical, *J. Am. Chem. Soc. 112*, 6995–7000.
8. Yamauchi, R., Matsui, T., Kato, K., and Ueno, Y. (1990) Reaction Products of α-Tocopherol with Methyl Linoleate-Peroxyl Radicals, *Lipids 25*, 152–158.

9. Liebler, D.C., and Burr, J.A. (1992) Oxidation of Vitamin E During Iron-Catalyzed Lipid Peroxidation: Evidence for Electron-Transfer Reactions of the Tocopheroxyl Radical, *Biochemistry 31*, 8278–8284.
10. Yamauchi, R., Yagi, Y., and Kato, K. (1994) Isolation and Characterization of Addition Products of α-Tocopherol with Peroxyl Radicals of Dilinoleoylphosphatidylcholine in Liposomes, *Biochim. Biophys. Acta 1212*, 43–49.
11. Durckheimer, W., and Cohen, L.A. (1964) The Chemistry of 9-Hydroxy-Alpha-Tocopherone, a Quinone Hemiacetal, *J. Am. Chem. Soc. 86*, 4388–4393.
12. Marcus, M.F., and Hawley, M.D. (1970) Electrochemical Studies of the Redox Behavior of Alpha-Tocopherol, *Biochim. Biophys. Acta 201*, 1–8.
13. Liebler, D.C., Kaysen, K.L., and Kennedy, T.A. (1989) Redox Cycles of Vitamin E: Hydrolysis and Ascorbic Acid Dependent Reduction of 8a-(alkyldioxy)Tocopherones, *Biochemistry 28*, 9772–9777.
14. Burton, G.W., and Ingold, K.U. (1981) Autoxidation of Biological Molecules. 1. The Antioxidant Activity of Vitamin E and Related Chain-Breaking Phenolic Antioxidants *in vitro*, *J. Am. Chem. Soc. 103*, 6472–6477.
15. Niki, E., Tanimura, R., and Kamiya, Y. (1982) Oxidation of Lipids. II. Rate of Inhibition of Oxidation by Alpha-Tocopherol and Hindered Phenols Measured by Chemiluminescence, *Bull. Chem. Soc. Jpn. 55*, 1551–1555.
16. Liebler, D.C., Kaysen, K.L., and Burr, J.A. (1991) Peroxyl Radical Trapping and Autoxidation Reactions of Alpha-Tocopherol in Lipid Bilayers, *Chem. Res. Toxicol. 4*, 89–93.
17. Liebler, D.C., Burr, J.A., Matsumoto, S., and Matsuo, M. (1993) Reactions of the Vitamin E Model Compound 2,2,5,7,8-Pentamethylchroman-6-ol with Peroxyl Radicals, *Chem. Res. Toxicol. 6*, 351–355.
18. Urano, S., Hattori, Y., Yamanoi, S., and Matsuo, M. (1980) ^{13}C Nuclear Magnetic Resonance Studies on Alpha-Tocopherol and its Derivatives, *Chem. Pharm. Bull. 28*, 1992–1998.
19. Boozer, C.E., Hammond, G.S., Hamilton, C.S., and Sen, J.N. (1955) Air Oxidation of Hydrocarbons. II. The Stoichiometry and Fate of Inhibitors in Benzene and Chlorobenzene, *J. Am. Chem. Soc. 77*, 3233–3237.
20. Pryor, W.A., Strickland, T., and Church, D.F. (1988) Comparison of the Efficiencies of Several Natural and Synthetic Antioxidants in Aqueous Sodium Dodecyl Sulfate Micelle Solutions, *J. Am. Chem. Soc. 110*, 2224–2229.
21. Niki, E., Takahashi, M., and Komuro, E. (1986) Antioxidant Activity of Vitamin E in Liposomal Membranes, *Chem. Lett.*, 1573–1576.
22. Barclay, L.R., Bailey, A.M., and Kong, D. (1985) The Antioxidant Activity of Alpha-Tocopherol-Bovine Serum Albumin Complex in Micellar and Liposome Autoxidations, *J. Biol. Chem. 260*, 15809–15814.
23. Horswill, E.C. and Ingold, K.U. (1966) The Oxidation of Phenols. I. The Oxidation of 2,6-Di-*t*-Butyl-4-Methylphenol, 2,6-Di-*t*-Butylphenol, and 2,6-Dimethylphenol with Peroxy Radicals, *Can. J. Chem. 44*, 263–268.

[Received August 1, 1994, and in revised form April 21, 1995; Revision accepted June 16, 1995]

Cholesterol Interaction with Recombinant Human Sterol Carrier Protein-2

S.M. Colles[a,1], J.K. Woodford[a,2], D. Moncecchi[a,3], S.C. Myers-Payne[b,4], L.R. McLean[c], J.T. Billheimer[d] and F. Schroeder[a,b,*]

[a]Division of Pharmacology and Medicinal Chemistry, Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0004, [b]Department of Physiology and Pharmacology, Texas Veterinary Medical Center, Texas A&M University, College Station, Texas 77843-4466, [c]Department of Analytical and Structural Sciences, Marion Merrell Dow Research Institute, Cincinnati, Ohio 45215-6300, [d]Cardiovascular Department, Du Pont Merck Pharmaceutical Company, Wilmington, Delaware 19898-0400

ABSTRACT: The interaction of human recombinant sterol carrier protein-2 (SCP-2) with sterols was examined. Two independent ligand binding methods, Lipidex 1000 binding of [^{3}H]cholesterol and a fluorescent dehydroergosterol binding assay, were used to determine the affinity of SCP-2 for sterols. Binding analysis indicated SCP-2 bound [^{3}H]cholesterol and dehydroergosterol with a K_d of 0.3 and 1.7 μM, respectively, and suggested the presence of a single binding site. Phase fluorometry and circular dichroism were used to characterize the SCP-2 sterol binding site. Alterations in dehydroergosterol lifetime, SCP-2 tryptophan lifetime, and SCP-2 tryptophan quenching by acrylamide upon cholesterol binding demonstrated a shielding of the SCP-2 tryptophan from the aqueous solvent by bound sterol. Differential polarized phase fluorometry revealed decreased SCP-2 tryptophan rotational correlation time upon cholesterol binding. Circular dichroism of SCP-2 indicated that cholesterol elicited a small decrease in SCP-2 alpha helical content. The data suggest that SCP-2 binds sterols with affinity consistent with a lipid transfer protein that may act either as an aqueous carrier or at a membrane surface to enhance sterol desorption.
Lipids 30, 795–803 (1995).

Sterol carrier protein-2 (SCP-2), also referred to as nonspecific lipid transfer protein, is one of several lipid binding proteins that are believed to be involved in intracellular cholesterol trafficking (1,2), steroid hormone biosynthesis (3), and bile acid synthesis (4). SCP-2 dramatically stimulates enzymatic reactions involving cholesterol esterification and steroid synthesis *in vitro* (1,2,4). These reactions include the *in vitro* microsomal conversion of intermediates between lanosterol and cholesterol (5), microsomal synthesis of cholesterol esters by acyl CoA–cholesterol acyl transferase (ACAT) (6), and conversion of mitochondrial cholesterol to steroid hormones (3,7). The role of SCP-2 in transferring cholesterol in intact cells was first demonstrated by transient co-expression of human SCP-2 cDNA with expression vectors for cholesterol side-chain cleavage enzyme and adrenodoxin in COS-7 cells. Expression of human SCP-2 resulted in a severalfold enhancement of progestin synthesis as compared to expression of the endogenous steroidogenic enzyme system alone (8).

The precise mechanism by which SCP-2 enhances these reactions of sterol metabolism is not known. The effect of SCP-2 on these reactions does not appear to be due to direct interaction of SCP-2 with the respective enzymes. At least four mechanisms have been proposed where SCP-2 may increase delivery of sterol to the site of reaction—aqueous carrier (2,9,10); tertiary complex formation between SCP-2, the donor, and the acceptor (11); direct membrane effect to enhance sterol desorption from the donor membrane (1); and membrane fusion (1,12). Although SCP-2 mediated membrane fusion has definitively been discounted (12), resolution of the remaining mechanism(s) remains.

The main objective of this study is to elucidate and characterize a potential cholesterol binding site in SCP-2. This is a significant question as SCP-2 purified from liver does not contain any bound cholesterol or other lipid (12,13). It is not known whether bound cholesterol is lost upon purification of SCP-2 or whether SCP-2 does not have a cholesterol binding site. The demonstration of such a site would be consistent with either the aqueous carrier mechanism, as well as a direct membrane effect mechanism whereby SCP-2 binds cholesterol in the membrane and increases its desorption from the membrane.

*To whom correspondence should be addressed at the Department of Physiology and Pharmacology, Texas A&M University, TVMC, College Station, TX 77843-4466.

[1]Present Address: Cleveland Clinic Foundation, 9500 Euclid Ave., NC10, Cleveland, OH 44195

[2]Present Address: The Andrew Jergens Co., 2535 Spring Grove Ave., Cincinnati, OH 44214-1773.

[3]Present Address: 6103 Browning Dr., Cheyenne, WY 82007.

[4]Present Address: Laboratory of Membrane Biochemistry and Biophysics, NIAAA, National Institutes of Health, FLOW Bldg., Rm. 55A, 12501 Washington St., Rockville, MD 20852.

Abbreviations: ACAT, acyl–CoA cholesterol acyltransferase; CD, circular dichroism; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; nsLTP, nonspecific lipid transfer protein; PS, phosphatidyl-L-serine; PC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SCP-2, sterol carrier protein-2; SUV, small unilamellar vesicles.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Materials. Phosphatidyl-L-serine (PS) and 1-palmitoyl-2-oleoyl phosphatidylcholine (PC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Dehydroergosterol ($\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol) was synthesized and its purity checked by high-performance liquid chromatography (HPLC) as previously described (14). Cholesterol (Sigma Chemical Co., St. Louis, MO) was recrystallized and its purity established by HPLC (14).

Recombinant human SCP-2 was isolated as described previously (15). Protein was by Bradford assay (Bio-Rad Laboratories, Richmond, CA) with bovine albumin as standard. All other chemicals were reagent grade or better.

Fluorescent sterol binding assay. The binding affinity of SCP-2 for dehydroergosterol was determined. Dehydroergosterol (0.2–2.0 μM) was added from a concentrated ethanolic stock solution to a sample of SCP-2 (0.6 or 1.0 μM) in 10 mM K_2HPO_4 (pH 7.4) at 24°C. The sample was continuously stirred and the final concentration of ethanol was ≤1.6%. Relative fluorescence intensity was measured upon reaching equilibrium (5 min). The sample was excited at 324 nm using an ISS PC1 photon counting spectrofluorimeter with a 450 watt xenon-arc lamp (I.S.S. Inc., Champaign, IL). Fluorescence emission was measured through a GG-375 sharp cut-off filter (Janos Technology Inc., Townshend, VT). Fluorescence from ligand-only controls were subtracted from those where protein was present to give ΔF. The data from three experiments, each with an n of 2 or 3, were plotted and fit with a simple rectangular hyperbola binding isotherm:

$$\Delta F = F_{max}[L]/(K_d + [L]) \quad [1]$$

where F_{max} is the maximum specific fluorescence enhancement determined from increasing protein added to a fixed, low concentration of ligand and plotted as a simple hyperbolic fit. $[L]$ is the concentration of free dehydroergosterol determined from a plot of increasing ligand added to a fixed concentration of protein. The concentration of free dehydroergosterol is calculated by subtracting the ΔF from the F_{max}. The K_d is defined as the value on the X axis at 50% of F_{max}.

Radiolabeled cholesterol binding assay. The binding of [1,2-^{3}H(N)]cholesterol (specific activity, 50.6 Ci/mmol; New England Nuclear, Boston, MA) to human recombinant SCP-2 was measured by the Lipidex-1000 assay (16) optimized for measuring sterol binding to SCP-2: The assays were carried out in polypropylene tubes with 0.4 mL 10 mM potassium phosphate, pH 7.4, buffer. SCP-2 was 0.4 μM and cholesterol was 0.04 to 1.2 μM (0.95% final ethanol concentration). SCP-2 was added to the assay tubes with mixing, and incubations were carried out at 37°C for 15 min, followed by cooling on ice for 10 min. Unbound cholesterol was removed by addition of 0.5 mL of a continuously-stirred Lipidex-1000 suspension, 50% vol/vol, in phosphate buffer. After a 10-min incubation on ice, with vortexing at 0, 5, and 10 min, the samples were centrifuged at 12,000 × *g* for 3 min. A 0.5-mL aliquot was removed and scintillation counted in an LS 7000 Scintillation Counter (Beckman Instruments Inc., Fullerton, CA). The data were plotted as a Scatchard plot with (*r*/[free cholesterol]) as the Y axis and ([bound/total sterol]) as the X axis. The concentration of bound cholesterol is derived from the % of bound value. The parameter *r* equals [bound sterol/total SCP-2]. The slope = $-1/K_d$ and the B_{max} is indicated by the X intercept.

Fluorescence lifetime determination. Samples consisted of 5 μM SCP-2 in previously described buffer (17) with or without 2 μM cholesterol. Fluorescence lifetime of dehydroergosterol was determined as described earlier (18,19). Self-quenching artifacts were avoided by diluting dehydroergosterol 1:10 with cholesterol. Light scatter was eliminated with a 375 nm emission cutoff filter (Janos Technology). SCP-2 tryptophan fluorescence lifetime was obtained with the same instrumentation, except that excitation at 305 nm was from an Innova 306 Argon Ion laser (Coherent Inc., Palo Alto, CA) with deep ultraviolet multiline optics. A 300-nm rejection filter (Andover Corp., Salem, NH) was used to block the 275 nm output line from the laser. Light scatter was reduced with UV34 (Schott Glass Technologies Inc., Duryea, PA) and U340 (Hoya Optics Inc., Fremont, CA) emission band pass filters. Fluorescence lifetime data were analyzed as described earlier (19,20). The reduced X^2 parameter was utilized to evaluate the "quality of fit" to the applied lifetime model. The data were fit to one, two, and three component lifetime models. A two-component fit (Table 1) was optimal and provided the lowest chi-square values and random residuals. Tryptophan differential polarized phase studies were performed as described earlier (21), except that excitation was with the argon ion laser.

Circular dichroic (CD) spectra. CD spectra of samples in 1-mm circular cuvettes were recorded at room temperature with a J-500A Spectropolarimeter (JASCO, Easton, MD) at human recombinant SCP-2 concentrations of 82 and 190 μg/mL. The CD spectrum of air was subtracted from the CD spectrum of the sample after each scan. The scan rate was 2 nm/min, the time constant was 8 s, and the slit width was 2 nm. Data were collected at 0.04-nm intervals and averaged over a 0.2-nm interval. The instrument was calibrated with a

TABLE 1
Dehydroergosterol Fluorescence Lifetime upon Interaction with SCP-2[a]

SCP-2 (μM)	τ1 (ns)	α1	τ2 (ns)	α2
0	0.73 ± 0.16	0.42 ± 0.03	3.14 ± 0.20	0.58 ± 0.03
5	0.80 ± 0.08	0.61 ± 0.02[d]	3.51 ± 0.16[b]	0.39 ± 0.02[d]
10	0.83 ± 0.01	0.73 ± 0.03[d,e]	3.80 ± 0.06[b,c]	0.27 ± 0.03[d,e]

[a]Samples consisting of dehydroergosterol (0.2 μM) and cholesterol (1.8 μM) were excited at 325 nm, and the lifetime of dehydroergosterol was determined using phase and modulation and analyzed by linear least squares analysis. τ1, τ2 represent the lifetime component ± SEM (n = 3), and α1, α2 represent the mole fraction ± SEM (n = 3) attributed to each lifetime. Samples were analyzed to obtain a minimal $\chi 2$.
[b]$P \leq 0.05$ vs. control using unpaired Student's *t*-test.
[c]$P \leq 0.05$ vs. 5 μM human recombinant SPC-2 using unpaired Student's *t*-test.
[d]$P \leq 0.005$ vs. control using unpaired Student's *t*-test.
[e]$P \leq 0.005$ vs. 5 μM human recombinant SCP-2 using unpaired Student's *t*-test.

0.6% solution of ammonium camphor sulfonate ($[\Theta]_{290.5}$ = 7910 deg cm^2/dmol; JASCO, Inc., Easton, MD). Calculations of secondary structure were made essentially as described previously (22), with constrained parameters and the reference data of Chen *et al.* (23) and Bolotina *et al.* (24) on data to 195 nm.

Steady-state fluorescence. Emission spectra and acrylamide quenching experiments were conducted at the University of Illinois, Laboratory for Fluorescence Dynamics (Urbana, IL), with a PC1 photon counting fluorometer (ISS Inc.). Samples were excited at 295 nm using a (300W) Xe-arc lamp. Emission spectra of 5 μM human recombinant SCP-2 were obtained alone or with either 2 μM cholesterol or 2 μM dehydroergosterol present. The emission spectra of distilled water, 2 μM cholesterol, and 2 μM dehydroergosterol were obtained for background subtraction. Absorbance was below 0.15 in all cases to avoid the inner filter effect. Light scatter was minimized by use of dilute solutions and through use of appropriate cutoff filters as described earlier.

SCP-2 tryptophan quenching experiments were conducted by addition of increasing amounts of acrylamide to 5 μM human recombinant SCP-2, either with or without 20 μM cholesterol. The corrected fluorescence intensities were analyzed using the Stern-Volmer equation as modified by Lehrer (25). The modified equation is valid for systems containing two populations of tryptophan: accessible and inaccessible to the quencher. A plot of $F_0/\Delta F$ vs. 1/[acrylamide] gives a y-intercept of $1/f_a$ and a slope equal to $1/f_aK$, where F_0 is the intensity in the absence of acrylamide, ΔF is the change in intensity at each point from F_0, f_a is the fraction of tryptophan accessible to the acrylamide, and K is the Stern-Volmer constant of the accessible tryptophan.

Fluorescent sterol exchange assay. Small unilamellar vesicles (SUV) were prepared as described earlier (19,20,26) with two modifications. First, donor SUV (PC/PS/dehydroergosterol, 55:10:35 mol%) and acceptor SUV (PC/PS/cholesterol, 55:10:35 mol%) were sonicated for 4 min until optical clarity was attained. Phospholipid was measured according to Ames (27). Second, the buffer (10 mM PIPES/0.02% NaN_3, pH 7.4) was prefiltered with a 0.20 μm filter (Millipore, Bedford, MA). The exchange of dehydroergosterol between donor and acceptor membranes was monitored at 22°C in the absence and presence of SCP-2 as described previously by this laboratory (19,20,28).

ACAT. Microsome isolation and ACAT assay were performed as previously described (29), except that preincubation was for 15 min and the incubation was for 10 min. Liposomes and SCP-2, when included, were added to the preincubation system.

RESULTS

Sterol binding to human recombinant SCP-2. Because purified SCP-2 does not contain bound ligands and as SCP-2 transfers both sterols and phospholipids, sterol binding assays without potential competing phospholipid ligands were used to examine binding to SCP-2. Because dehydroergosterol as aqueous monomers has very low quantum yield, observed fluorescence was due to micellar dehydroergosterol. Upon titration of SCP-2 with dehydroergosterol, the fluorescence intensity due to bound dehydroergosterol (corrected for intensity of dehydroergosterol blanks) increased (Fig. 1). Scatchard analysis of the data gave a K_d of 1.7 ± 0.7 μM (n = 7) and a B_{max} of 0.9 mole/mole, which would suggest a single dehydroergosterol binding site.

To eliminate the possibility that the observed binding of dehydroergosterol by SCP-2 might not be the same as cholesterol, a Lipidex-1000 radioligand binding assay, originally developed for fatty acid binding to fatty acid binding proteins, was modified to examine binding of [^{3}H]cholesterol to SCP-2. As shown in Figure 2, Scatchard analysis of [^{3}H]cholesterol binding to SCP-2 yielded a K_d = 0.31 ± 0.05 μM (n = 3).

Effect of SCP-2 on dehydroergosterol lifetime. Fluorescence lifetime analysis of a dehydroergosterol with or without SCP-2 was used to determine if the sterol interacted with SCP-2. In the absence of SCP-2, dehydroergosterol had a short lifetime component at 0.73 ns and a longer lifetime component at 3.14 ns (Table 1). Dehydroergosterol monomers in aqueous solution have extremely low quantum yield and were not detected. The shorter lifetime component represents dehydroergosterol in a more nonpolar environment while the longer lifetime component represents dehydroergosterol more aqueous exposed within the aqueous dehy-

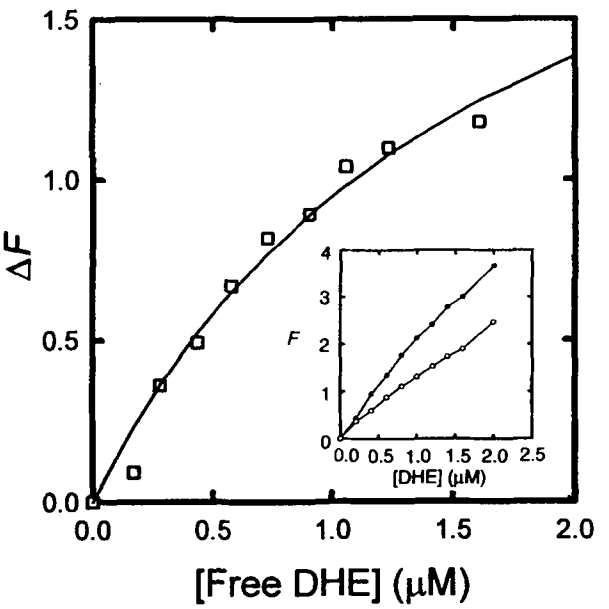


FIG. 1. Dehydroergosterol (DHE) binding by human recombinant sterol carrier protein-2 (SCP-2). Data points represent the specific fluorescence enhancement as DHE by human SCP-2 (1.0 μM) and have been corrected for background fluorescence due to DHE alone. The curve is a theoretical fit to a single binding site isotherm using the equation $\Delta F = F_{max}[L]/(K_d + [L])$. In this particular experiment, the predicted F_{max} is 3.0 units, giving a K_d of 2.0 μM. The standard error was 0.56 and had a dependency of 0.987. In the inset, the fluorescence enhancement of DHE alone (○) and DHE plus SCP-2 (●) are plotted. The difference is ΔF.

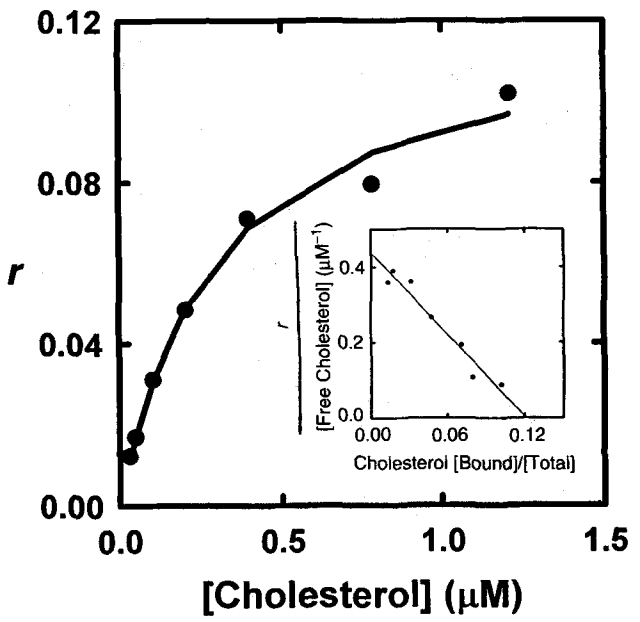


FIG. 2. Binding of radiolabeled cholesterol to human recombinant SCP-2. The parameter *r* equals [bound sterol/total SCP-2]. A plot of *r* vs. cholesterol concentration shows increasing binding of cholesterol by human recombinant SCP-2. The inset shows a Scatchard plot of *r*/[free cholesterol] μM^{-1} vs. [bound/total] cholesterol. Values represent the average of two assays each with an n = 2. Abbreviation as in Figure 1.

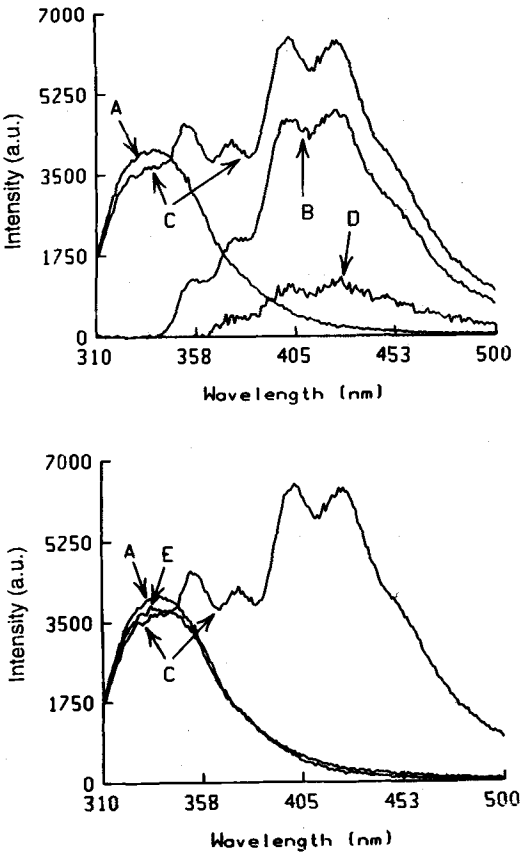


FIG. 3. Fluorescence emission spectra of human SCP-2 samples with or without either cholesterol or dehydroergosterol. Spectra were obtained as described in the Materials and Methods section with excitation at 295 nm. The top panel shows spectra obtained in the presence of dehydroergosterol. Line A is human SCP-2, B is dehydroergosterol, C is human SCP-2 plus dehydroergosterol, and D is line C minus lines A and B. The bottom panel shows line A, which is human SCP-2, line E is human SCP-2 plus cholesterol, and line C is human SCP-2 plus dehydroergosterol. Abbreviation as in Figure 1.

droergosterol micelles. The mole fraction of dehydroergosterol represented by each of the micellar components was 0.42 and 0.58, respectively (Table 1). Addition of human recombinant SCP-2 altered the fractional contributions of the two lifetime components of dehydroergosterol. In the presence of human recombinant SCP-2, almost twofold more dehydroergosterol (0.73 mole fraction) became localized within a nonpolar environment. Concomitantly, the second lifetime component increased significantly by 0.66 ns in the presence of 10 μM human recombinant SCP-2 (Table 1).

Second, human recombinant SCP-2 contains a single tryptophan. Tryptophan emission (Fig. 3, top panel, curve A) overlaps with dehydroergosterol excitation (not shown). If the two molecules have an optimal orientation factor, good overlap of emission of SCP-2 tryptophan, and dehydroergosterol absorbance and are in close proximity within the sterol binding site of human recombinant SCP-2, Forster nonradiative energy transfer occurs from tryptophan donor to dehydroergosterol acceptor. The emission spectra of dehydroergosterol, human recombinant SCP-2 alone, and human recombinant SCP-2 in the presence of dehydroergosterol were obtained with excitation of the single SCP-2 tryptophan at 295 nm. Because of the broad excitation spectrum of dehydroergosterol (Fig. 3, line B), there was also excitation of dehydroergosterol at the tryptophan excitation wavelength. It was not possible to excite tryptophan in the complete absence of exciting dehydroergosterol. However, the excitation of dehydroergosterol at 295 is considerably less than that of tryptophan. The emission spectrum of SCP-2 in the presence of dehydroergosterol was examined for alterations consistent with energy transfer from the tryptophan residue of human recombinant SCP-2 to dehydroergosterol. The top panel of Figure 3 shows emission scans for samples containing dehydroergosterol. All scans were background subtracted using distilled water as the blank. When human recombinant SCP-2 was incubated with dehydroergosterol, fluorescence intensity of dehydroergosterol near 400 nm increased (Fig. 3, top panel, line C) compared to dehydroergosterol without human recombinant SCP-2 (Fig. 3, top panel, line B). Concomitantly, in the presence of dehydroergosterol, the fluorescence intensity of SCP-2 tryptophan near 331 nm decreased (Fig. 3, top panel, line C) compared to SCP-2 alone (Fig. 3, top panel, line A). Subtraction of the human recombinant SCP-2 and dehydroergosterol spectra from line C indicated that the increased intensity that occurred was greater than can be explained by simple overlap of their emission spectra (Fig. 3, top panel, line D). Furthermore, the increase in sensitized emission of dehydroergosterol was accompanied by a concomitant decrease in tryptophan emission intensity for human recombinant SCP-2 tryptophan near 331 nm in the presence of dehydroergosterol (Fig. 3, top panel,

curve C vs. A). These results are consistent either with energy transfer, a conformational change in the human recombinant SCP-2 upon sterol binding, or an alteration of the tryptophan environment upon human recombinant SCP-2 tryptophan binding sterol. The latter could occur if the tryptophan were exposed to some aqueous solvent in a binding pocket of the apoprotein and shielded in the holoprotein when the hydrophobic sterol occupies the binding pocket. These three possibilities were examined in the following experiments.

Comparison of energy transfer and conformational changes in human recombinant SCP-2 upon ligand binding. To prove that the decreased SCP-2 tryptophan fluorescence intensity in the presence of dehydroergosterol (Fig. 3, top panel, curve C vs. A) was due to energy transfer, it is necessary to show the absence of any changes in tryptophan emission spectrum upon addition of a nonfluorescent sterol, cholesterol. However, cholesterol quantitatively affected the tryptophan emission spectrum similarly (Fig. 3, bottom panel, curve A vs. E), to dehydroergosterol (Fig. 3, top panel, curve C vs. A). Because cholesterol is nonfluorescent and does not absorb significantly in the region of tryptophan emission, energy transfer was considered unlikely. Thus, energy transfer from tryptophan to dehydroergosterol can be eliminated as a possible explanation. However, the lack of energy transfer does not preclude the possibility that the tryptophan and dehydroergosterol are still in close proximity. Lack of energy transfer could be due to an unfavorable orientation of the tryptophan rather than the tryptophan being located distant from the dehydroergosterol.

Acrylamide quench of tryptophan fluorescence. The effect of sterol binding on the exposure of SCP-2 tryptophan to the aqueous environment was examined with the water-soluble quencher acrylamide. The tryptophan residue of human recombinant SCP-2, with or without cholesterol present, was excited at 295 nm while increasing amounts of acrylamide were added. Changes in tryptophan fluorescence emission intensity at 331 nm were shown as modified Stern-Volmer plots (Fig. 4). The quenching plots of tryptophan in human recombinant holo-SCP-2 were linear, indicating the presence of a single type of tryptophan residues. The low modified Stern-Volmer quenching constant in the absence of cholesterol (modified K_{SV} = 4.19 M) indicated poor accessibility of this tryptophan residue to the water-soluble acrylamide. A small shift toward the baseline (decreased slope) was observed in the presence of cholesterol. This indicated that the tryptophan was even less accessible to quenching by acrylamide when cholesterol was bound to human recombinant SCP-2. This is further illustrated by the modified K_{SV} values for the quenching. The addition of cholesterol decreased the modified K_{SV} value from 4.19 M to 2.22 M, consistent with decreased quenching upon cholesterol binding. This would suggest a shielding of SCP-2 tryptophan by bound sterol and/or a modest conformational change occurring in the human SCP-2 protein upon cholesterol binding.

Effect of cholesterol on human recombinant SCP-2 tryptophan fluorescence lifetime. Based on these changes in tryptophan accessibility to quenching acrylamide, it is predicted that the tryptophan lifetime also should be altered if a conformational change occurred in the SCP-2. Human recombinant SCP-2 tryptophan fluorescence lifetime was examined in the presence and absence of cholesterol. The SCP-2 tryptophan emission was best resolved into two components near 2.34 and 0.53 ns (Table 2). The mole fractions attributed to these components were 0.33 and 0.67, respectively. Upon addition of 2 μM cholesterol, there was a significant 0.51 ns increase in the lifetime of the long lifetime component ($P \leq 0.05$). There was no significant redistribution of the mole fractions associated with each lifetime component. The increase in tryptophan lifetime upon binding of sterol suggested that the SCP-2 altered its conformation slightly to orient the tryptophan in a more nonpolar environment.

Differential polarized phase fluorimetry of human recombinant SCP-2 tryptophan best fit two rotational correlation components (32.2 and 2.8 ns) describing an ellipsoidal shape. Furthermore, upon addition of cholesterol, there was a de-

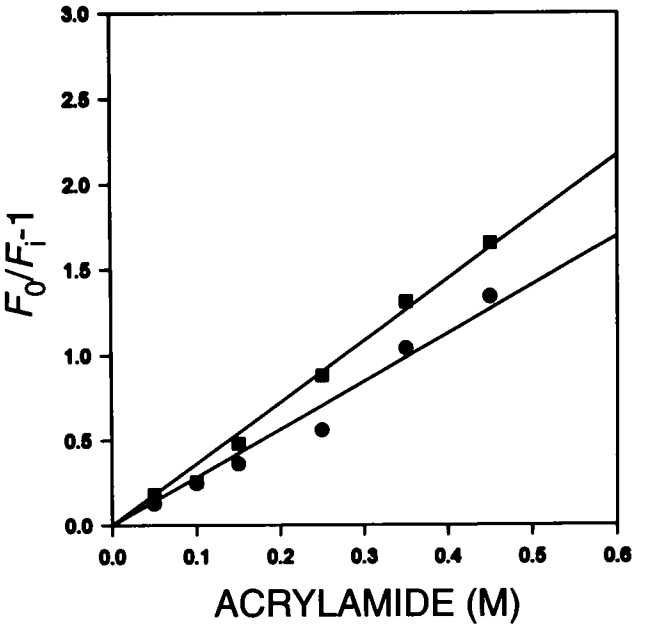


FIG. 4. Acrylamide quench experiments of human SCP-2 with or without cholesterol. Experiments were performed as described in the Materials and Methods section. The open circles (○) represent human SCP-2 and the closed circles (●) represent human SCP-2 plus cholesterol. Abbreviation as in Figure 1.

TABLE 2
Effects of Cholesterol on SCP-2 Tryptophan Fluorescence Lifetime Emission[a]

Cholesterol (μM)	τ1 (ns)	α1	τ2 (ns)	α2
0	2.34 ± 0.15	0.33 ± 0.05	0.53 ± 0.05	0.67 ± 0.05
2	2.85 ± 0.13[b]	0.26 ± 0.07	0.63 ± 0.02	0.74 ± 0.03

[a]Samples of human recombinant SCP-2 (5 μM) with or without cholesterol were excited at 295 nm, and the lifetime of tryptophan emission was determined using phase and modulation and analyzed by linear least squares analysis. Data were handled in a similar manner as explained in the Materials and Methods section and Table 1. Abbreviations as in Table 1.
[b]$P \leq 0.05$ using unpaired Student's *t*-test (n = 6).

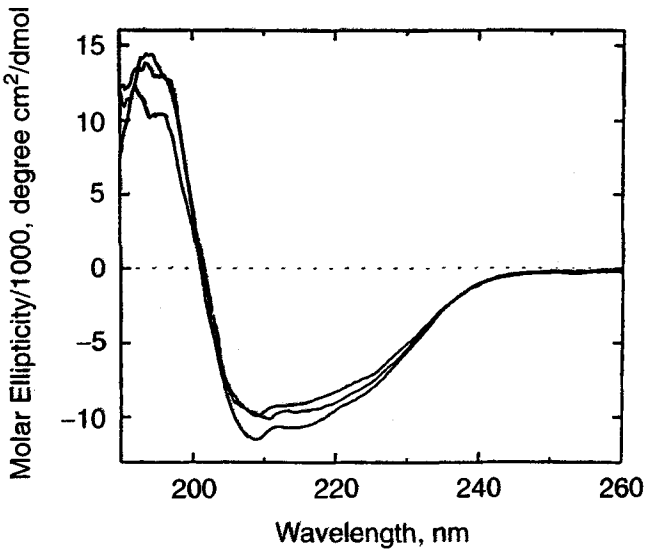


FIG. 5. Effect of cholesterol on dehydroergosterol on circular dichroism spectra of human SCP-2. Spectra were obtained for 6 μM human SCP-2 in a 20 mM phosphate buffer at pH 7.4 in the absence and presence of either 2 μM cholesterol or 2 μM dehydroergosterol. The spectra are ordered at 210 nm from bottom to top: no additions, plus dehydroergosterol, plus cholesterol.

crease in the rotational correlation time for both the long and the short axes, to 21.4 and 2.1 ns, respectively. This was consistent with a conformational change in human recombinant SCP-2 upon interaction with sterol.

CD studies of the conformational properties of human recombinant SCP-2. The effect of sterol binding on human SCP-2 conformation was examined directly through CD. In the far ultraviolet without added ligands, the CD spectra of human SCP-2 (Fig. 5) displayed minima at 208 and 222 nm, consistent with substantial α-helical content. Analysis of the spectrum revealed 31 ± 1% α-helix (Table 3) in the absence of ligands. Analysis of CD spectra by the methods of either Chen *et al.* or Bolotina *et al.* (see Materials and Methods section) both yielded virtually identical values for α-helical content of human SCP-2, 31 ± 1 vs. 30.5 ± 0.5, respectively. Decreasing the concentration of protein in solution did not alter the shape of the spectrum or the fitted secondary structural parameters. A twofold decrease in human recombinant SCP-2 altered the α-helical % content of human recombinant SCP-2 less than 1%. Thus, the SCP-2 was not aggregated, and small differences in protein content could not account for the 3–3.5% decreased α-helical content of human recombinant SCP-2 upon addition of cholesterol (Table 3).

Functional activity of human recombinant SCP-2. Although this data suggested that sterols bind to human recombinant SCP-2, such binding alone does not signify that the protein is actually involved in sterol transfer activity. Therefore, two assays were performed to show functional activity for the recombinant SCP-2.

First, the effect of human SCP-2 on sterol transfer between SUV, was examined using a fluorescence polarization assay which we recently developed (19,20,28,30). The spontaneous exchange kinetics of dehydroergosterol in model membranes were resolved into two exchangeable domains with half times near 18.7 and 185 min. The mole fraction of the exchangeable and nonexchangeable domains was 0.04, 0.83, and 0.13. Human recombinant SCP-2 (1.1 μM) stimulated intermembrane sterol transfer by dramatically decreasing the half times of exchange from 18.7 to 1.1 and from 185 to 98 min, respectively. Concomitantly, the mole fraction of the rapidly exchangable domain increased from 0.04 to 0.68, the slowly exchangable domain decreased from 0.83 to 0.32, and the nonexchangable domain was eliminated.

Second, the ability of human recombinant SCP-2 to enhance cholesterol esterification *in vitro* was determined. The human recombinant SCP-2 increased the formation of cholesterol ester from 145 ± 16 in the absence of SCP-2 to 452 ± 22 in the presence of 10 μg SCP-2 as described in the Materials and Methods section. This stimulation was similar to that noted with native rat liver SCP-2 (15).

DISCUSSION

Both ligand binding and spectroscopic data suggest that SCP-2 interacts with the fluorescent sterol, dehydroergosterol, and cholesterol. The low intrinsic solubility of sterols in aqueous buffer has complicated demonstration of cholesterol binding to SCP-2, and potential binding assays are at the limit of applicability. These assays use micellar substrates and contain significant amounts of solvents. Nevertheless, two such assays were used to examine the possibility of sterol binding to SCP-2.

First, a fluorescence assay using dehydroergosterol was developed to demonstrate binding of a sterol to the human re-

TABLE 3
Analysis of Circular Dichroism Spectra of Human Sterol Carrier Protein-2 with and without Cholesterol

	Chen analysis[a]			Bolotina analysis[b]			
Cholesterol (μM)	α	β	Random	α	β	Turn	Random
0	31 ± 1	33 ± 0	36 ± 1	31.5 ± 0.5	19 ± 0	20 ± 0	29.5 ± 0.5
0.5	28	36	36	28	20	21	31
2.0	28	34	38	28	19	21	32

[a]Analysis according to Chen *et al.* (Ref. 23). Values with ± represent mean ± SEM (n = 3). α, Alpha-helix; β, beta-sheet.
[b]Analysis according to Bolotina *et al.* (Ref. 24). Values with ± represent mean ± SEM (n = 3).

combinant SCP-2. Although a high degree of unspecific fluorescence of dehydroergosterol in the absence of SCP-2 was observed, this type of assay has the advantage of not requiring separation of bound from free ligand. As demonstrated by fluorescence intensity, dehydroergosterol bound to human recombinant SCP-2 with 1:1 stoichiometry and K_d of 1.7 μM.

Second, although dehydroergosterol is an excellent fluorescent analogue whose structure and many other properties mimic those of cholesterol more closely than other known cholesterol probe molecules (reviewed in Ref. 9), it is still possible that the affinity of SCP-2 for dehydroergosterol does not reflect that of cholesterol. Therefore, a Lipidex 1000 assay with [^{3}H]cholesterol was used to confirm this observation. In order to optimize conditions for cholesterol binding, the assay was modified to contain about tenfold higher Lipidex 1000 resin than used for fatty acid binding. [^{3}H]Cholesterol was bound by human recombinant SCP-2 with K_d of 0.3 μM. It was not possible to accurately obtain a B_{max} with the Lipidex 1000 assay due to the very low solubility of cholesterol (20 nM critical micellar concentration), which precluded saturation of the human recombinant SCP-2 cholesterol binding site prior to precipitation of large cholesterol aggregates. The finding that human recombinant SCP-2 bound [^{3}H]cholesterol suggests that its binding of dehydroergosterol was not just a unique feature of the fluorescent sterol. However, SCP-2 did appear to bind cholesterol about sevenfold more tightly than dehydroergosterol, perhaps due to assay difference.

Several sets of spectroscopic results were consistent with a close molecular interaction of sterol with SCP-2 rather than nonspecific absorption of SCP-2 to micellar sterol. Firstly, dehydroergosterol lifetime analysis showed two components—a short lifetime component near 0.73 associated with a nonpolar microenvironment, and a longer lifetime component associated with a more aqueous exposed environment in the dehydroergosterol micelle. Two similar lifetimes and microenvironments were observed for dehydroergosterol in membranes (18). SCP-2 did not alter either lifetime in the dehydroergosterol micelle; it did increase the fraction of shorter lifetime component in hydrophobic environment. This suggests that the hydrophobicity of the SCP-2 dehydroergosterol binding site is similar to that within the nonpolar region of the dehydroergosterol micelle. Second, tryptophan emission decreased upon interaction with sterols. Acrylamide quenching and tryptophan lifetime studies further suggested that this decrease was not due to energy transfer but to shielding of SCP-2 tryptophan by bound sterol. Third, both rotational correlation time and CD studies indicated that SCP-2 undergoes a modest conformational change upon interaction with sterol.

The finding that human recombinant SCP-2 binds sterols addresses a significant unresolved issue. Although it is generally agreed that SCP-2 enhances sterol transfer between membranes, the issue of whether SCP-2 actually binds cholesterol is unclear. This is an important point as SCP-2 purified from liver does not contain bound sterol (12,13). The discrepancy appears to result from the substrate form and/or assay used. When sterol is the only potential ligand in the assay system, binding of sterol to SCP-2 is observed. For example, light scatter studies showed that native rat liver SCP-2 binds dehydroergosterol with 1:1 stoichiometry and 1.4–1.7 μM K_d (21). The Lipidex-1000 assay was used to show that chicken liver SCP-2 (also called nonspecific lipid transfer protein, nsLTP) bound [^{3}H]cholesterol (31). Although it might be argued that presentation of the sterol substrate in micellar form (21,31) is nonphysiological, other studies with adrenal neutral lipid droplets, a physiological substrate form (4,13), are also consistent with cholesterol binding to SCP-2 (32). Adrenal lipid droplets contain primarily neutral lipid (81% cholesteryl ester, 10% triglyceride, 3% cholesterol, and 6% phospholipid) (13). The adrenal lipid droplets were incubated with SCP-2, the subnatant fraction was reisolated, and mass of bound ligand was determined (4). SCP-2 bound cholesterol (but not triglyceride, cholesteryl esters, or phospholipids) with 1:1 stoichiometry. Finally, although the sterol binding observed is not a unique property of SCP-2, it is not an aspecific protein effect. For example, another cytosolic lipid transfer protein, the liver fatty acid binding protein, also binds dehydroergosterol and [^{3}H]cholesterol (18). However, the closely related cytosolic intestinal cytosolic fatty acid binding protein does not bind either dehydroergosterol or [^{3}H]cholesterol (18). Consistent with this, chicken liver nsLTP, but not chicken liver fatty acid binding protein, binds [^{3}H]cholesterol (31). In summary, these types of studies suggest that SCP-2 can bind sterol.

In contrast to these studies with a single potential binding substrate, one study under conditions where the sterol is incorporated into model membranes reported phospholipid, but not sterol, binding to SCP-2 (17). However, both phospholipids and dehydroergosterol were transferred between model membranes by the nsLTP (17). This is in contrast to another study which concluded that SCP-2 bound and transferred cholesterol, but not phospholipid, between natural donors and acceptors (13). In any case, the model membrane assay for dehydroergosterol and fluorescent phosphoplipid binding to nsLTP has several limitations. First, the model membranes had multiple potential ligands (phosphatidylcholine, pyrene-labeled phosphatidylcholine, trinitrophenyl-phosphatidylethanolamine) in addition to dehydroergosterol. SCP-2 binds phospholipids (17,32–34). If the phospholipids and sterols compete for the same binding site on SCP-2, then in this very complicated system (four competing substrates) the relative affinity of SCP-2 for the different phospholipids vs. sterol will determine the degree of respective ligand loading in the SCP-2. Second, the model membrane assay was complicated by the use of multiple quenching molecules (17). Not only was trinitrophenyl-phosphatidylethanolamine used to quench dehydroergosterol emission in the donor membrane, but the dehydroergosterol concentration used (10–50 mole%) was self-quenching. Previous work from our laboratory showed that the dehydroergosterol is self-quenched in model membranes containing more than 6 mole% dehydroergosterol (26). As a consequence, it was reported that dehydroergosterol transfer increased 13-fold and did not compete with the pyrene-labeled phospholipid when dehydroergosterol content

of the donor model membranes was increased from 10–50 mole%. In contrast, through use of a different dehydroergosterol transfer assay (with only a single quenching process), our laboratory showed that the half-times of dehydroergosterol transfer in phosphatidylcholine vesicles containing 10 and 35 mole% (20), as well as 50 mole% (35), did not differ significantly. These data were consistent with those wherein a standard assay with [^{3}H]cholesterol in model membranes was used and no difference in cholesterol transfer half-time between 10 and 35 mole% cholesterol was observed (20). Third, the lack of dehydroergosterol binding from neutral model membrane vesicles does not represent a physiological membrane situation. Biological membranes contain significant amounts of negatively charged phospholipids. SCP-2 binds negatively charged phospholipids up to 13-fold better (17) and low amounts of anionic phospholipids (e.g., 10 mole%) enhance the ability of SCP-2 to transfer sterol between membranes up to several orders of magnitude (9,20,28,30,36). Fourth, in the aforementioned study (17), negatively charged phospholipids were present in the pyrene-phospholipid-SCP-2 binding assay, but not in the dehydroergosterol-SCP-2 binding assay. Thus, the relative binding ability of SCP-2 for phospholipid and cholesterol was not examined under equivalent conditions. Consequently, the SCP-2 did not have equivalent access to sterol and phospholipid ligands in the donor membrane. Fifth, the use of phospholipid model membranes presents SCP-2 with multiple, potentially competing, ligand sites (membrane and SCP-2). Not only will the affinity of the SCP-2 for the respective phospholipids and sterol determine extent of binding, but the affinity of the membrane itself for the respective phospholipids and sterol will complicate demonstration of the binding of sterol to SCP-2. Sixth, the use of pyrene-labeled lipids to mimic the behavior of native lipids is subject to intrinsic structural differences between the probe and the native molecule. For example, pyrene labeled sterol analogues had 4.4-fold faster exchange rates than did radiolabeled cholesterol (37). In contrast, dehydroergosterol is a naturally-occurring fluorophore (9), replaces up to 90% of endogenous sterol in cultured cells without altering cell functions and lipid composition (9), and transfers with half-times not significantly different from those of [^{3}H]cholesterol (20).

In addition to demonstrating binding of sterols to human recombinant SCP-2, the present investigation reports some of the characteristics of such a binding site. The data suggest that SCP-2 binds sterols and that sterol binding shields the SCP-2 tryptophan from the aqueous medium. In addition, lifetime, rotational correlation time and CD studies further suggest that the SCP-2 undergoes a modest conformational change upon cholesterol binding. The finding that cholesterol significantly increased the human recombinant SCP-2 % of α-helical content by 3–3.5% is consistent with a report that phospholipid binding to wheat nsLTP increases the % of α-helical content by 8% (38). The suggestion, based on rotational relaxation time, that SCP-2 is ellipsoidal, is consistent with nuclear magnetic resonance (NMR) data on the structure of human recombinant SCP-2 (39). Although NMR structure of the human recombinant apo-SCP-2 (39) and the wheat apo-lipid transfer protein (40) have been reported, they did not address the lipid binding site. However, a site directional mutagenic approach (41) showed that amino acid residues between 100 and 104 are essential to human recombinant SCP-2 sterol transfer activity. Thus, a putative sterol binding site at the carboxyl terminal of SCP-2 may be far removed from the amino terminal amphipathic basic helix thought to be the membrane binding domain (39,40,42). Indeed, comparison to the NMR-based three-dimensional structure of human recombinant SCP-2 indicates that these residues (strand 5 of the beta sheet) appear to be located at the opposite face of and near one end of the SCP-2 molecule at a distance removed from the amphipathic-helical, basic amino terminal (39). A more precise determination from the NMR data is not possible pending NMR or X-ray crystallographic data on cholesterol-containing holo-SCP-2.

In conclusion, the results presented here and by others are consistent with at least two possible mechanisms whereby SCP-2 may enhance sterol transfer between membranes. In both cases, SCP-2 interacts with the membrane, binds sterol, and a sterol-SCP-2 complex desorbs from the membrane. Then either (i) the desorbed sterol-SCP-2 complex is transient and quickly releases sterol into the aqueous medium, or (ii) the SCP-2 acts as an aqueous carrier between donor and acceptor membranes. Both possibilities would require the presence of a sterol binding site in the protein and formation of a soluble, even if transient, lipid SCP-2 complex. In the case of the desorption model, the binding site may be weak and not easily detected. The half-time of the sterol bound to the SCP-2 could be extremely short. The latter would account for the very low saturation of the binding site observed when phospholipid membranes are the source of sterol (17). Recently, kinetic evidence of fluorescent phospholipid transfer by bovine liver nsLTP is consistent with the formation of a soluble lipid-nsLTP complex being the key step in nsLTP-mediated phospholipid transfer (43). Additional evidence shows that SCP-2 does not act as an aqueous carrier (44), but instead binds to membranes and acts to enhance sterol desorption. An SCP-2 sterol binding site is required for this process (44).

ACKNOWLEDGMENTS

This work was supported in part by the National Institute of Health (GM31651). S.M.C. was supported in part by a postdoctoral fellowship from an NIH Training Grant (HL07527).

REFERENCES

1. Billheimer, J.T., and Reinhart, M.P. (1990) *Subcellular Biochemistry: Intracellular Transfer of Lipid Molecules* (Hilderson, H.J., ed.) Vol. 16, pp. 301–331, Plenum Press, New York.
2. Moncecchi, D., Nemecz, G.N., Schroeder, F., and Scallen, T.J. (1991) *Physiology and Biochemistry of Sterols* (Patterson, G.W., and Nes, W.D., eds.) pp. 1–27, American Oil Chemists' Society, Champaign.
3. Pfeiffer, S.M., Furth, E.E., Ohba, T., Chang, Y.J., Rennert, H., Sakuragi, N., Billheimer, J.T., and Strauss, J.F. (1993) *J. Steroid Biochem. Molec. Biol. 47*, 167–172.

4. Scallen, T.J., Pastuszyn, A., Noland, B.J., Chanderbhan, R., Kharroubi, A., and Vahouny, G.V. (1985) *Chem. Phys. Lip. 38*, 239–261.
5. Noland, B.J., Arebalo, R.E., Hansbury, E., and Scallen, T.J. (1980) *J. Biol. Chem. 255*, 4282–4289.
6. Gavey, K.L., Noland, B.J., and Scallen, T.J. (1981) *J. Biol. Chem. 256*, 2993–2999.
7. Vahouny, G.V., Chanderbhan, R., Noland, B.J., Irwin, D., Dennis, P., Lambeth, J.D., and Scallen, T.J. (1983) *J. Biol. Chem. 258*, 11731–11737.
8. Yamamoto, R., Kallen, C.B., Babalola, G.O., Rennert, H., Billheimer, J.T., and Strauss, J.F. (1991) *Proc. Natl. Acad. Sci. USA. 88*, 463–467.
9. Schroeder, F., Jefferson, J.R., Kier, A.B., Knittel, J., Scallen, T.J., Wood, W.G., and Hapala, I. (1991) *Proc. Soc. Exper. Biol. Med. 196*, 235–252.
10. Butko, P., Hapala, I., Scallen, T.J., and Schroeder, F. (1990) *Biochemistry* 29, 4070–4077.
11. Van Amerongen, A., Teerlink, T., Van Heusden, G.P.H., and Wirtz, K.W.A. (1985) *Chem. Phys. Lip. 38*, 195–204.
12. Crain, R.C., and Zilversmit, D.B. (1980) *Biochemistry 19*, 1433–1439.
13. Chanderbhan, R., Noland, B.J., Scallen, T.J., and Vahouny, G.V. (1982) *J. Biol. Chem. 257*, 8928–8934.
14. Fischer, R.T., Stephenson, F.A., Shaifee, A., and Schroeder, F. (1985) *J. Biol. Phys. 13*, 13–24.
15. Matsuura, J.E., George, H.J., Ramachandran, N., Alvarez, J.G., Strauss III, J.F., and Billheimer, J.T. (1993). *Biochemistry 32*, 567–572.
16. Glatz, J.F.C., and Veerkamp, J.H. (1983) *Anal. Biochem. 132*, 89–95.
17. Gadella, T.W., and Wirtz, K.W.A. (1991) *Biochim. Biophys. Acta 1070*, 237–245.
18. Nemecz, G., and Schroeder, F. (1991) *J. Biol. Chem. 266*, 17180–17186.
19. Schroeder, F., and Nemecz, G. (1989) *Biochemistry 28*, 5992–6000.
20. Nemecz, G., Fontaine, R.N., and Schroeder, F. (1988). *Biochim. Biophys. Acta 943*, 511–521.
21. Schroeder, F., Butko, P., Nemecz, G., and Scallen, T.J. (1990) *J. Biol. Chem. 265*, 151–157.
22. McLean, L.R., and Hagaman, K.A. (1989) *Biochemistry 28*, 321–327.
23. Chen, Y.H., Yang, J.T., and Chan, K.H. (1974) *Biochemistry 13*, 3350–3359.
24. Bolotina, I.A., Chekhov, V.O., Lugauskas, V.Iu., Finkel'shtein, A.V., and Ptitsyn, O.B. (1980) *Mol. Biol. (Moscow, USSR) 14*, 891–901.
25. Lehrer, S.S. (1971) *Biochemistry 10*, 3254–3263.
26. Schroeder, F., Barenholz, Y., Gratton, E., and Thompson, T.E. (1987) *Biochemistry 26*, 2441–2448.
27. Ames, B.N. (1968) *Methods Enzymol. 8*, 115–118.
28. Butko, P., Hapala, I., Nemecz, G., and Schroeder, F. (1992) *J. Biochem. Biophys. Meth. 24*, 15–34.
29. Billheimer, J.T., Tavani, D., and Nes, W.R. (1981) *Anal. Biochem. 111*, 331–335.
30. Hapala, I., Kavecansky, J., Butko, P., Scallen, T.J., Joiner, C.H., and Schroeder, F. (1994) *Biochemistry 33*, 7682–7690.
31. Sams, G.H., Hargis, B.M., and Hargis, P.S. (1991) *Comp. Biochem. Physiol. 99B*, 213–219.
32. Gadella, T.W.J., Bastiaens, P.I.H., Visser, A.J.W.G., and Wirtz, K.W.A. (1991) *Biochemistry 30*, 5555–5564.
33. Nichols, J.W. (1987) *J. Biol.Chem. 162*, 14172–14177.
34. Nichols, J.W. (1988) *Biochem. 27*, 1889–1896.
35. Kavecansky, J., Joiner, C.H., and Schroeder, F. (1994) *Biochem. 33*, 2880–2890.
36. Nemecz, G.N., and Schroeder, F. (1988) *Biochemistry 22*, 7740–7749.
37. McLean, L.R., and Phillips, M.C. (1981) *Biochem. 20*, 2893–2900.
38. Desormeaux, A., Blochet, J.-E., Pezolet, M., and Marion, D. (1992) *Biochim. Biophys. Acta 1121*, 1019–1028.
39. Seedorf, U., Scheek, S., Engel, T., Steif, C., Hinz, H-J., and Assman, G. (1994) *J. Biol. Chem. 269*, 2613–2618.
40. Gincel, E., Simorre, J.-P., Caille, A., Marion, D., Ptak, M., and Vovelle, F. (1994) *Eur. J. Biochem. 226*, 413–422.
41. Szyperski, T., Scheek, S., Johansson, J., Assmann, G., Seedorf, U., and Wuthrich, K. (1993) *FEBS Lett. 335*, 18–26.
42. Pastuszyn, A., Noland, B.J., Bazan, F., Fletterick, R.J., and Scallen, T.J. (1987) *J. Biol. Chem. 262*, 13219–13227.
43. Gadella, T.W.J., and Wirtz, K.W.A. (1994) *Eur. J. Biochem. 220*, 1019–1028.
44. Woodford, J.K., Colles, S.M., Myers-Payne, S., Billheimer, J.T., and Schroeder, F. (1995) *Chem. Phys. Lip. 76*:73–84.

[Received July 13, 1994, and in final revised form May 1, 1995; Revision accepted July 5, 1995]

Molecular Species of Sphingomyelin in Sphingomyelinase-Sensitive and Sphingomyelinase-Resistant Pools of HL-60 Cells

Veronica Fitzgerald, Merle L. Blank and Fred Snyder*

Medical Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee 37831-0117

ABSTRACT: This study of sphingomyelin molecular species in undifferentiated and differentiated (granulocytic form) HL-60 cells demonstrated only minor differences in the distribution of species between the sphingomyelinase-sensitive and sphingomyelinase-resistant pools of sphingomyelin in these cells. The two most prominent species of sphingosine present in both the undifferentiated and differentiated cells were those containing 16:0 (slightly higher in the sphingomyelinase-resistant membranes) and 24:1 *N*-acyl moieties. Cell differentiation exerted little effect on the distribution of molecular species of sphingomyelin between the sphingomyelinase-sensitive and sphingomyelinase-resistant pools in HL-60 cells, although the levels of *N*-palmitoyl sphinganine were significantly lower and the *N*-nervonoyl sphingosine higher in both pools from the differentiated cells. Our results indicate the same species of sphingomyelin, available at both the outer layer of the plasma membrane and inner layer of the plasma membrane (plus intracellular membranes) of HL-60 cells, serve as precursors for generation of the ceramides that participate in signal transduction processes initiated by cell activation.

Lipids 30, 805–809 (1995).

Recent research has indicated that sphingomyelin, in addition to being a structural component of cellular membranes, is involved in signal transduction processes through the generation of ceramides by the action of a sphingomyelinase (1–10). Several investigators have utilized HL-60 cells as the biological system for their studies (1–4,9,10). Yet as far as we are aware, the molecular species of sphingomyelin in HL-60 cells have not been analyzed. Determination of molecular species of sphingomyelin would seem to take on greater importance after a recent report suggesting that a specific "signalling" pool of sphingomyelin is resistant to treatment with sphingomyelinase (11) in HL-60 cells. The question then arises as to whether this precursor pool of sphingomyelin may consist of different types of molecular species than present in the more inert pool of sphingomyelin associated with the outer portion of the plasma membrane. We therefore investigated the possibility of whether a specific distribution of sphingomyelin molecular species in the membranes of HL-60 cells could contribute to the difference in biological activity associated with the sphingomyelinase-sensitive and sphingomyelinase-resistant pools of sphingomyelin in these cells.

*To whom correspondence should be addressed at Medical Sciences Division, Oak Ridge Associated Universities, P.O. Box 117, Oak Ridge, TN 37831-0117.

Abbreviations: GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; TLC, thin-layer chromatography; UV, ultraviolet.

MATERIALS AND METHODS

Cell culture. Human leukemic cells (HL-60; purchased from the American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 media containing 10% heat inactivated fetal calf serum, 100 units of penicillin per mL, 100 μg of streptomycin per mL, 2 mM L-glutamine, and 20 mM HEPES buffer. Cells were maintained in a humidified, 5% CO_2 atmosphere at 37°C. Stock cultures were usually diluted 1:4 with fresh media when they reached a cell density of 1.5×10^6 cells/mL. Cells to be treated with sphingomyelinase were isolated from stock cultures by centrifugation at $200 \times g$ for 15 min and washed twice with Dulbecco's phosphate buffered saline (PBS) with the same centrifugation between washes. The washed cells were resuspended in the same media (0.4×10^6 cells/mL) used for stock cultures except the fetal calf serum was replaced with insulin (10 μg/mL), transferrin (10 μg/mL), sodium selenite (100 μg/mL), and fatty acid free bovine serum albumin (0.1% wt/vol). Cells were then incubated in the serum-free media for 48 h, pelleted, washed once with PBS, resuspended in serum-free media (1.6×10^6 cells/mL), and incubated 2 h before adding 0.1 unit of sphingomyelinase (*Staphlococcus aureus*) per mL of media (11). An identical volume of the sphingomyelinase vehicle (0.25 M phosphate buffer pH 7.4 in glycerol/water, 1:1) was added to control cultures. After 20 min in the presence of the sphingomyelinase at 37°C, the cells were pelleted by centrifugation at $600 \times g$ for 10 min and the cellular lipids extracted (12). These procedures are nearly identical to those used previously by others to hydrolyze the sphingomyelin in the outer layer of the plasma membrane of HL-60 cells (9,11). A similar approach also has been used to study the asymmetric distribution of sphingomyelin molecular species in human ery-

Copyright © 1995 by AOCS Press

throcyte membranes (13). The validity of using sphingomyelinase to determine the external and internal amounts of sphingomyelin also has been demonstrated recently on hepatocytes (14).

Some HL-60 cells were also differentiated into granulocytic type cells by adding dimethyl sulfoxide to the media (15) six days prior to the sphingomyelinase treatment. The average extent of cellular differentiation was 77.7 ± 5.5% (mean ± SE) as measured by the intracellular reduction of nitro blue tetrazolium (16).

Determination of sphingomyelin hydrolysis by sphingomyelinase. Phospholipids from sphingomyelinase-treated cells and vehicle-treated control cells were separated by thin-layer chromatography (TLC) on plates coated with silica gel H that were developed in chloroform/methanol/glacial acetic acid/water (50:25:8:4, by vol). The developed plates were sprayed with sulfuric acid, charred by heating, and the amount of phosphorus in each phospholipid class was measured (17). The percentage decrease in sphingomyelin between the controls and sphingomyelinase-treated cells was taken as the percent of sphingomyelin that had been hydrolyzed.

Preparation of benzoate derivatives. Cellular lipid extracts were divided in half, and one portion was benzoylated using benzoic anhydride and dimethylaminopyridine as previously described for diradylglycerols (18). Ceramide dibenzoates, representing the sphingomyelin that was originally associated with the outer leaflet of the plasma membrane (11), were isolated from these reaction products by preparative TLC on silica gel G plates developed in hexane/diethyl ether (1:1, vol/vol). The developed plates were sprayed with a solution of 0.05% 2,7-dichlorofluorescein in 50% ethanol and viewed under ultraviolet (UV) light to locate the ceramide dibenzoate band (R_f ~ 0.3) which was extracted (12). Sphingomyelinase-resistant sphingomyelin was isolated from the second half of the total lipid extract by TLC on silica gel H plates developed in chloroform/methanol/glacial acetic acid/water (25:15:4:3, by vol). Developed plates were exposed to ammonia fumes for ~ 3 min and then sprayed with 0.05% 2,7-dichlorofluorescein in absolute ethanol to locate the sphingomyelin band under UV light. The sphingomyelin was extracted from the silica gel by the method of Bligh and Dyer (12) and subjected to phospholipase C hydrolysis (19,20) to remove the phosphocholine group. Total products from this phospholipase C hydrolysis were benzoylated and the ceramide dibenzoates isolated by TLC as described earlier.

Analysis of ceramide dibenzoates by high-performance liquid chromatography (HPLC). The identical dual pump HPLC system (Beckman Instruments, Berkeley, CA) previously used for separation of diradylglycerolbenzoate molecular species (20) was used to separate the molecular species of the dibenzoate derivatives of ceramides. A mobile phase of acetonitrile/isopropanol (3:2, vol/vol) at a flow rate of 1 mL per min and a detector set at 230 nm was used for the ceramide dibenzoate analyses.

A ceramide dibenzoate standard was prepared from beef brain ceramides (Sigma Chemical Co., St. Louis, MO) and purified by TLC as described in a previous section. The purified dibenzoate derivative had a molar absorptivity of 2.62 x 10^4 at 230 nm, which would be the expected value for a dibenzoate derivative (20). According to earlier work, treatment of ceramides with benzoic anhydride leads to *O*-benzoylation but not to *N*-benzoylation (21). The components of 13 different HPLC peaks from the beef brain ceramide dibenzoates were collected and treated with HCl–methanol at 70°C for 18 h to produce the free sphingoid bases and methyl esters (22). These products were benzoylated (18,20), and the resulting mixture of methyl esters and benzoate derivatives of the sphingoid bases was separated by TLC on silica gel G plates developed in hexane/diethyl ether (1:1, vol/vol). The developed plates were sprayed with 0.05% 2,7-dichlorofluorescein in 50% ethanol, viewed under UV light to locate the benzoylated sphingoid bases (R_f ~ 0.3) and methyl esters (R_f ~ 0.7), which were then extracted (12) from the silica gel. Addition of 2–3 drops of ammonium hydroxide during this extraction results in almost no contamination of dichlorofluorescein in the lower chloroform layer. The methyl esters were then analyzed by gas–liquid chromatography (GLC), and the sphingoid benzoates were separated by HPLC. An elution solvent of acetonitrile:isopropanol (4:1, vol/vol) at a flow rate of 1 mL per min through a 250 mm x 4.6 mm column packed with 5 μM Ultrasphere-ODS (Beckman Instruments) caused the sphingosine tribenzoate to be eluted at 7.9 min and sphinganine tribenzoate at 9.4 min. On the basis of graphical plots of log retention time vs. chain length the sphingosine (purchased from Sigma Chemicals) contained small amounts of 16:1 and 20:1 (<10%) bases in addition to the expected 18:1 sphingosine base. These analyses permitted identification of 13 major peaks in the beef brain ceramide benzoate standard (tentative identification of other minor peaks were extrapolated through the linear relationship that exists between log retention time and chain lengths). The composition of the beef brain ceramide benzoates was similar to that reported previously using slightly different methodology (23).

Identification of sphingomyelin molecular species in samples from HL-60 cells was based primarily on comparison of HPLC retention times of the ceramide dibenzoate derivatives between the samples and the standard prepared from beef brain. In addition, four of the larger separated ceramide dibenzoate fractions from undifferentiated HL-60 cells were also collected from the HPLC separation and subjected to the analysis of *N*-acyl and sphingoid base groups as described for the beef brain ceramides.

RESULTS AND DISCUSSION

Although HL-60 cells do contain low amounts of ceramide (1,2), it was calculated from information on the amounts of sphingomyelin in HL-60 cells (24) that sphingomyelin levels are about five times higher than the ceramide contents. Therefore, the small amount of free ceramides present in HL-60 cells would not likely cause a problem in interpreting our results. Also, based on the fact that no sphingosine and very lit-

tle sphingomyelin (2.8% in 12 h) was produced by HL-60 cells labeled with [^{3}H]acetylsphingosine (2), any changes in the distribution of ceramide species caused by further metabolism of ceramides during the sphingomyelinase treatment would appear to be minimal.

The extent of sphingomyelin hydrolysis by treatment of HL-60 cells with sphingomyelinase for 20 min was 56%; this accessible pool is considered to be on the outside surface of the cells. The nanomoles of sphingomyelin per 10^7 cells were 10.32 and 4.52 before and after treatment, respectively. Our results agree well with the previously reported amount of sphingomyelinase-accessible sphingomyelin (60%) present in the outer leaflet of the plasma membranes from HL-60 cells (11) using a slightly different analytical technique. TLC analysis of lipids extracted from the media of HL-60 cells after treatment with sphingomyelinase revealed only barely detectable amounts of ceramides, indicating that the ceramide portion of the sphingomyelin molecule remained associated with the outer plasma membrane after sphingomyelinase treatment. The distribution of molecular species in ceramides obtained by either sphingomyelinase or phospholipase C treatment of total sphingomyelin isolated from undifferentiated HL-60 cells was identical (data not shown). Thus, there was no change in the molecular species distribution that could be ascribed to a difference in selectivity between sphingomyelinase and phospholipase C treatments.

An example of the HPLC separation of ceramide dibenzoates, prepared by phospholipase C hydrolysis and benzoylation of the sphingomyelin that remained after treatment of HL-60 cells with sphingomyelinase, is shown in Figure 1. The largest component (peak A), identified as the dibenzoate of *N*-palmitoyl sphingosine, makes up about 50 mole% of the total species. The molecular species eluting with peak E contains nervonic acid and was the next most abundant (~16 mole %) component of the sphingomyelin in HL-60 cells. Identification of peaks A, B, E, and G were verified to contain at least 90% of the indicated molecular species by GLC analysis of their *N*-acyl groups (as methyl esters) and HPLC separation of the benzoate derivatives of the sphingoid bases as described in the Materials and Methods section. Identification of other peaks is based solely on their retention times from HPLC.

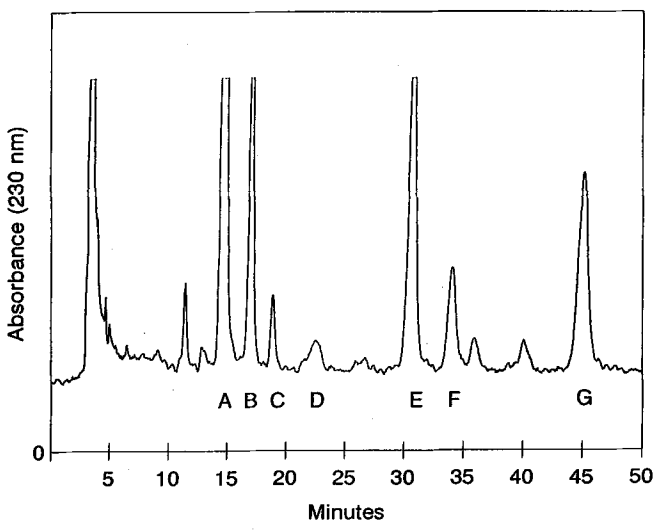


FIG. 1. High-performance liquid chromatography tracing of ceramide dibenzoate derivatives prepared as described in the Materials and Methods section from the sphingomyelin located on the outer membrane surface (sphingomyelinase-sensitive) of undifferentiated HL-60 cells. Full-scale absorbance at 230 nm for this chromatogram was 0.013 O.D. units. See Tables 1 and 2 for identification of the molecular species associated with letters located below the peaks.

Table 1 shows the distribution of molecular species of sphingomyelin that were sensitive (outside) or resistant (inside) to sphingomyelinase treatment of undifferentiated HL-60 cells. Small but statistically significant differences in percentages between the sphingomyelinase-sensitive (outside) and sphingomyelinase-insensitive (inside) sphingomyelin of undifferentiated HL-60 cells were seen in molecular species eluting with benzoate derivatives of *N*-palmitoyl (higher on the inside) and *N*-lignoceroyl sphingosine (higher on the outside, Table 1). However, we did not observe the extensive asymmetric distribution of sphingomyelin molecular species in HL-60 cells that is present in human erythrocyte membranes (13). Except for a small difference in the amount of the *N*-palmitoyl sphinganine species, the differentiated HL-60 cells did not show a pronounced difference in distribution of sphingomyelin molecular species between the sphingomyelinase-sensitive and sphingomyelinase-resistant membrane pools (Table 2). However, the amount of *N*-palmitoyl sphinganine was significantly lower ($P < 0.02$), and the level of *N*-nervonoyl sphingosine was significantly higher ($P < 0.02$) in differentiated cells than was found in the sphingomyelin from both sphingomyelinase-sensitive and sphingomyelinase-resistant pools of sphingomyelin from undifferentiated cells. These data indicate that, although differentia-

TABLE 1
Distribution of Sphingomyelin Species Between the Sphingomyelinase-Sensitive and Sphingomyelinase-Resistant Pools in Membranes of Undifferentiated HL-60 Cells[a]

Peak	*N*-acyl group	Inside (mole percent)	Outside (mole percent)
A	16:0	54.0 ± 1.4	47.5 ± 1.6[b]
B	16:0[c]	11.4 ± 2.3	9.4 ± 1.3
C	18:0	3.2 ± 0.2	3.2 ± 0.5
D	22:1	2.7 ± 0.2	2.6 ± 0.2
E	24:1	16.1 ± 1.4	16.5 ± 0.8
F	22:0	3.8 ± 0.3	4.2 ± 0.2
G	24:0	6.0 ± 0.7	9.5 ± 0.7[d]

[a]Values are the average percent (± SE from three separate experiments, each with duplicate samples) of either the molecular species hydrolyzed by sphingomyelinase treatment of the intact HL-60 cells (outside) or those that were resistant to the sphingomyelinase (inside). Other species were also present but none at levels above 2.5 mol%. Determinations of statistically significant differences in the distribution of species between the inside and outside surfaces were based on Student's *t*-test for paired variables ([b]$P < 0.05$ and [d]$P < 0.01$). High-performance liquid chromatography retention times correspond to those of ceramide dibenzoate species with the indicated *N*-acyl groups connected to a sphingosine base, except for (*c*), in which the 16:0 *N*-acyl group is linked to a sphinganine base.

TABLE 2
Distribution of Sphingomyelin Species Between the Sphingomyelinase-Sensitive and Sphingomyelinase-Resistant Pools in Membranes of Differentiated HL-60 Cells[a]

Peak	*N*-acyl group	Inside (mole percent)	Outside (mole percent)
A	16:0	53.8 ± 0.7	51.8 ± 2.4
B	16:0[c]	6.5 ± 0.5	4.1 ± 0.3[b]
C	18:0	3.0 ± 1.2	2.4 ± 0.2
D	22:1	3.3 ± 0.7	4.0 ± 0.2
E	24:1	22.1 ± 0.5	23.6 ± 0.6
F	22:0	3.9 ± 0.4	4.0 ± 0.2
G	24:0	5.2 ± 0.7	6.8 ± 0.9

[a]Values are the average percent (± SE from three separate experiments) of either the molecular species hydrolyzed by sphingomyelinase (outside) or those that were resistant to the sphingomyelinase treatment (inside) in DMSO-induced differentiated cells. Other footnotes are the same as those described in Table 1.

TABLE 3
Distribution of Total Sphingomyelin Species in Undifferentiated and Differentiated HL-60 Cells[a]

Peak	*N*-acyl group	Undifferentiated (mole percent)	Differentiated (mole percent)
A	16:0	55.4 ± 0.8	56.4 ± 1.3
B[b]	16:0[c]	11.2 ± 1.2	6.0 ± 0.2
C[d]	18:0	2.9 ± 0.1	1.8 ± 0.1
D	22:1	2.3 ± 0.3	2.8 ± 0.5
E[b]	24:1	15.7 ± 1.0	19.4 ± 0.7
F	22:0	3.4 ± 0.2	3.6 ± 0.2
G	24:0	6.0 ± 0.7	4.8 ± 0.4

[a]Values are the average mole percent ± standard error. Other species also were present but none at levels above 2.5 mol%. Determinations of statistically significant differences in the distribution of species between the undifferentiated and differentiated cells were based on Student's *t*-test for paired variables ([b]$P < 0.05$ and [d]$P < 0.001$). High-performance liquid chromatography retention times correspond to those of ceramide dibenzoate species for the indicated *N*-acyl groups connected to a sphingosine base.
[c]The 16:0 *N*-acyl group for this fraction is linked to a sphinganine base.

tion of HL-60 cells to their granulocytic form does not alter the amount of cellular sphingomyelin (25), the molecular species are indeed modified by treatment with dimethyl sulfoxide.

Statistically significant differences also were observed in some molecular species of the total sphingomyelin between undifferentiated and differentiated HL-60 cells (Table 3) that served as controls (cells not treated with sphingomyelinase). In these controls, the sphingomyelin species containing the *N*-palmitoyl sphinganine base and *N*-stearoyl sphingosine base were significantly higher in the undifferentiated cells, and the sphingomyelin containing the *N*-nervonyl sphingosine base was significantly lower; no significant differences were seen in any of the other molecular species of sphingomyelin analyzed in these cells (Table 3).

There is not enough difference in the distribution of sphingomyelin molecular species between the outer leaflet of the HL-60 plasma membrane and the sphingomyelinase-resistant pool of sphingomyelin to suggest that a particular sphingomyelin species could be involved in the sphingomyelin cycle associated with the signalling events described recently (11). Our experiments, while not precluding this possibility, do demonstrate that if specific molecular species of intracellular sphingomyelin are involved in cell signalling events, then essentially the same species are available for selection on the metabolically active internal cellular membranes (11) as are present on the outer surface of the membrane leaflet.

We believe the techniques for analyses of molecular species of sphingomyelin described in this report will be very useful in future studies designed to establish the functional role of sphingomyelin in cells. Moreover, our data provide baseline information about the various molecular species of sphingomyelin in HL-60 cells, a cell line that has proven to be a useful model system in a variety of studies dealing with cell signalling phenomena.

ACKNOWLEDGMENTS

The authors express their sincere appreciation to Shirley L. Poston for her excellent assistance in the preparation of this manuscript. This work was supported by the Office of Energy Research, U.S. Department of Energy (Contract No. DE-AC05-760R00033).

REFERENCES

1. Okazaki, T., Bell, R.M., and Hannun, Y.A. (1989) Sphingomyelin Turnover Induced by Vitamin D_3 in HL-60 Cells. Role in Cell Differentiation, *J. Biol Chem. 264*, 19076–19080.
2. Okazaki, T., Bielawska, A., Bell, R.M., and Hannun, Y.A. (1990) Role of Ceramide as a Lipid Mediator of 1α, 25-Dihydroxyvitamin D_3-Induced HL-60 Cell Differentiation, *J. Biol. Chem. 265*, 15823–15831.
3. Kim, M-Y., Linardic, C., Obeid, L., and Hannun, Y.A. (1991) Identification of Sphingomyelin Turnover as an Effector Mechanism for the Action of Tumor Necrosis Factor α and γ-Interferon. Specific role in Cell Differentiation, *J. Biol. Chem. 266*, 484–489.
4. Mathias, S., Dressler, K.A., and Kolesnick, R.N. (1991) Characterization of a Ceramide-Activated Protein Kinase: Stimulation by Tumor Necrosis Factor α, *Proc. Natl. Acad. Sci.* USA 88, 10009–10013.
5. Goldkorn, T., Dressler, K.A., Muindi, J., Radin, N.S., Mendelsohn, J., Menaldino, D., Liotta, D., and Kolesnick, R.N. (1991) Ceramide Stimulates Epidermal Growth Factor Receptor Phosphorylation in A431 Human Epidermoid Carcinoma Cells, *J. Biol. Chem. 266*, 16092–16097.
6. Dobrowsky, R.T., and Hannun, Y.A. (1992) Ceramide Stimulates a Cytosolic Protein Phosphatase, *J. Biol. Chem. 267*, 5048–5051.
7. Dobrowsky, R.T., Kamibayashi, C., Mumby, M.C., and Hannun, Y.A. (1993) Ceramide Activates Heterotrimeric Protein Phosphatase 2A, *J. Biol. Chem. 268*, 15523–15530.
8. Obeid, L.M., Linardic, C.M., Karolak, L.A., and Hannun, Y.A. (1993) Programmed Cell Death Induced by Ceramide, *Science 259*, 1769–1771.
9. Jayadev, S., Linardic, C.M., and Hannun, Y.A. (1994) Identification of Arachidonic Acid as a Mediator of Sphingomyelin Hydrolysis in Response to Tumor Necrosis Factor α, *J. Biol. Chem. 269*, 5757–5763.
10. Jarvis, W.D., Kolesnick, R.N., Fornari, F.A., Traylor, R.S., Gewirtz, D.A., and Grant, S. (1994) Induction of Apoptotic

DNA Damage and Cell Death by Activation of the Sphingomyelin Pathway, *Proc. Natl. Acad. Sci.* USA *91*, 73–77.
11. Linardic, C.M., and Hannun, Y.A. (1994) Identification of a Distinct Pool of Sphingomyelin Involved in the Sphingomyelin Cycle, *J. Biol. Chem. 269*, 23530–23537.
12. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37*, 911–917.
13. Boegheim, J.P.J., Jr., Van Linde, M., Op Den Kamp, J.A.F., and Roelofsen, B. (1983) The Sphingomyelin Pools in the Outer and Inner Layer of the Human Erythrocyte Membrane Are Composed of Different Molecular Species, *Biochim. Biophys. Acta* 735, 438–442.
14. Shiao, Y-J., and Vance, J.E. (1993) Sphingomyelin Transport to the Cell Surface Occurs Independently of Protein Secretion in Rat Hepatocytes, *J. Biol. Chem. 268*, 26085–26092.
15. Breitman, T.R., Collins, S.J., and Keene, B.R. (1980) Replacement of Serum by Insulin and Transferrin Supports Growth and Differentiation of the Human Promyelocytic Cell Line, HL-60, *Exp. Cell Res. 126*, 494–498.
16. Chaplinski, T.J., and Niedel, J.E. (1982) Cyclic Nucleotide-Induced Maturation of Human Promyelocytic Leukemic Cells, *J. Clin. Invest. 70*, 953–964.
17. Rouser, G., Siakotos, A.N., and Fleischker, S. (1966) Quantitative Analysis of Phospholipids by Thin-Layer Chromatography and Phosphorus Analysis of Spots, *Lipids 1*, 85–86.
18. Blank, M.L., Cress, E.A., Fitzgerald, V., and Snyder, F. (1990) Thin-Layer and High-Performance Liquid Chromatographic Separation of Glycerolipid Subclasses as Benzoates. Derivatives of Ether and Ester Analogues of Phosphatidylcholine, Phosphatidylethanolamine and Platelet-Activating Factor, *J. Chromatogr. 508*, 382–385.
19. Mavis, R.D., Bell, R.M., and Vagelos, P.R. (1972) Effect of Phospholipase C Hydrolysis of Membrane Phospholipids on Membranous Enzymes, *J. Biol. Chem. 247*, 2835–2841.
20. Blank, M.L., Robinson, M., Fitzgerald, V., and Snyder, F. (1984) Novel Quantitative Method for Determination of Molecular Species of Phospholipids and Diglycerides, *J. Chromatogr. 298*, 473–482.
21. Sugita, M., Iwamori, M., Evans, J., McCluer, R.H., Dulaney, J.T., and Moser, H.W. (1974) High Performance Liquid Chromatography of Ceramides: Application to Analysis in Human Tissues and Demonstration of Ceramide Excess in Farber's Disease, *J. Lipid Res. 15*, 223–226.
22. Gaver, R.C., and Sweeley, C.C. (1965) Methods for Methanolysis of Sphingolipids and Direct Determination of Long-Chain Bases by Gas Chromatography, *J. Am. Oil Chem. Soc. 42*, 294–298.
23. Smith, M., Monchamp, P., and Jungalwala, F.B. (1981) Separation of Molecular Species of Sphingomyelin and Ceramide by Argentation and Reversed-Phase HPLC, *J. Lipid Res. 22*, 714–719.
24. Billah, M.M., Eckel, S., Myers, R.F., and Siegel, M.I. (1986) Metabolism of Platelet-Activating Factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by Human Promyelocytic Leukemic HL-60 Cells, *J. Biol. Chem. 261*, 5824–5831.
25. Suga, K., Kawasaki, T., Blank, M.L., and Snyder, F. (1990) An Arachidonoyl (polyenoic)-Specific Phospholipase A_2 (PLA_2) Activity Regulates the Synthesis of Platelet-Activating Factor (PAF) in Granulocytic HL-60 Cells, *J. Biol. Chem. 265*, 12363–12371.

[Received April 3, 1995, and in final revised form June 19, 1995; Revision accepted July 24, 1995]

Lipid Changes in HL-60 Cells on Differentiation into Macrophages by Treatment with a Phorbol Ester

Ray Manning[a], Anna Fallani[b] and Salvatore Ruggieri[b,*]

[a]Department of Biological Sciences, University of Durham, Durham DH1 3LE, England, and [b]Istituto di Patologia Generale dell'Università degli Studi di Firenze, Viale Morgagni 50, 50134 Firenze, Italy

ABSTRACT: We studied changes in lipid composition of human promyelocytic leukemia cells (HL-60) on differentiation to the macrophage/monocytic lineage by treatment with the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate. Differentiation was accompanied by: (i) a decrease in the level of phospholipids; (ii) a greater amount of triacylglycerols; (iii) an increase in 1-alk-1'-enyl-2-acyl- and 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine and a decrease in 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; and (iv) an increase in the level of arachidonic acid in ethanolamine phospholipids. The increased levels of ether-linked lipids and of arachidonic acid in ethanolamine phospholipids are consistent with an enhanced biosynthesis of platelet-activating factor and eicosanoids, which are particularly important in the macrophage function.
Lipids 30, 811–815 (1995).

In spite of the great deal of information on the lipid structure of mammalian cells, our knowledge of the biological roles of the various lipid components is still limited. In fact, there are only few examples of cell functions related to specific lipid molecules. Cell permeability is known to be controlled by cholesterol levels (1,2) and the fatty acid profile of membrane phospholipids (3), and the activity of several membrane-associated enzymes is dependent on specific phospholipid classes (4,5) and molecular species (6–11). Moreover, receptor activities (12,13), agglutinability by lectins (14,15), and cellular adhesiveness (16) were shown to be influenced by specific fatty acids and phospholipid molecular species. Furthermore, a surfactant activity has been attributed to lung dipalmitoyl phosphatidylcholine (17). Most of this information has been derived from studies based on dietary manipulations (6,10,13, 18,19), treatment with lipases (4,11,20), or incorporation of exogenous lipids into cell membranes (5,9,15,21,22).

An alternative approach to the investigation of the biological role of lipids is to study the lipid changes which occur in different types of cells during differentiation. The rationale for this approach is that a characteristic lipid pattern acquired during differentiation may reflect the appearence of specific cellular functions. Suitable experimental models for this particular approach are represented by transformed cells which differentiate in response to specific treatments, such as Friend erythroleukemia cells (23), myeloid leukemia cells (24), and HL-60 promyelocytic leukemia cells (25). HL-60 cells are a particularly useful model in this type of experiment as these cells can differentiate into either mature granulocytes (25) or macrophages (26), the functions of which are partly dependent on production of biologically-active lipids. Indeed, differentiation of HL-60 into macrophages was associated with an increased synthesis of platelet-activating factor (PAF) (27) and leukotriene (LT) B_4 (LTB_4) (28), whereas differentiation into granulocytes was accompanied by an enhanced generation of LTC_4, LTD_4 as well as LTB_4 (28).

In the present study, we investigated whether the differentiation of HL-60 cells into macrophages, caused by treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), was associated with changes in the lipid structure which might reflect the metabolic and functional characteristics typical of the mature macrophage. Some aspects of lipid composition of HL-60 cells differentiated into macrophages (29,30) and granulocytes (31–33) have been previously described.

MATERIALS AND METHODS

Cells and culture conditions. HL-60 cells supplied by the American Type Culture Collection (Rockville, MD) were kindly donated by Menarini Laboratorios (Barcelona, Spain). HL-60 cells were grown at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 5% (vol/vol) heat-inactivated fetal calf serum (FCS) (Boehringer, Mannheim, Germany) and 2 mM L-glutamine, without antibiotics. Cells were subcultivated by seeding 5×10^5 cells/mL in Falcon tissue culture flasks (Becton Dickinson Labware, Bedford, MA) and were harvested after six days. At this time, cultures showed a density of 1×10^6 cells/mL.

Differentiation of HL-60 cells by TPA. HL-60 cells were suspended at 1×10^6 cells/mL in the growth medium containing 0.1 μM TPA (Sigma Chemical Co., St. Louis, MO), and

*To whom correspondence should be addressed.

Abbreviations: CP, choline phospholipid(s); DMSO, dimethyl sulfoxide; DPG, diphosphatidylglycerol; EP, ethanolamine phospholipids; FCS, fetal calf serum; GLC, gas–liquid chromatography; LT, leukotriene; PA, phosphatidic acid; PBS, phosphate-buffered saline; PI + PS, phosphatidylinositol + phosphatidylserine; PL, phospholipid(s); SP, sphingomyelin; TG, triacylglycerols; TLC, thin-layer chromatography; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

Copyright © 1995 by AOCS Press

were seeded into 100-mm Falcon plastic dishes. The TPA concentration used in this study (0.1 μM) was observed to induce the highest number of adherent cells (65–70% of cells in the culture) without affecting cell viability. The final concentration of dimethyl sulfoxide (DMSO) used to solubilize TPA did not exceed 0.02% (vol/vol) in growth medium, a concentration that was not found to affect the differentiation of HL-60 cells (26). After a 24-h incubation at 37°C, growth medium was decanted, and the remaining adherent cells were gently washed with 10 mL of phosphate-buffered saline (PBS) at 37°C, and then harvested with a rubber policeman scraper. Cells were counted by an automatic cell counter (ZBI; Coulter Electronics, Luton, Beds., England), and viability was determined by the Trypan Blue exclusion test. Cells suspended in cold PBS supplemented with 10% (vol/vol) FCS at pH 7.4 were smeared onto glass slides and cytocentrifuged at 500 rpm for 5 min (Haereus-Minifuge GL, Osterode, Germany). Slides were stained with a Giemsa solution and used to assess the morphology of macrophage-like differentiated cells. Macrophage features included disappearence of azurophilic granules, a marked adherence to plastic with prominent pseudopodia and adherence of cells to each other. Differentiation into monocyte/macrophage lineage was also assessed by appearance of α-naphthylacetate esterase activity (34). Suspensions of adherent TPA-treated HL-60 cells were also tested for CD11b and CD11c markers, characteristic of monocytic/macro-phagic differentiation, using monoclonal antibodies (Becton Dickinson, Mountain View, CA) in an indirect immunofluorescence procedure. The expression of CD11b and CD11c markers was ten times higher in TPA-treated adherent HL-60 cells than in undifferentiated HL-60 cells (results not shown). Flow-cytometric analysis was performed on a FACS machine (Fluorescent Activated Cell Sorter; Becton Dickinson).

Lipid analysis. TPA-differentiated HL-60 cells were suspended in PBS and washed three times with PBS, and then extracted with 19 vol of chloroform/methanol (2:1, vol/vol) following the procedure of Folch *et al.* (35). This procedure also was used to extract lipids from undifferentiated HL-60 cells after they were freed from growth medium by centrifugation and resuspension in PBS.

Neutral lipids and phospholipids (PL) were fractionated into individual classes as reported previously (36). Triacylglycerols (TG) were determined by the quantitative gas–liquid chromatography (GLC) of their fatty acid methyl esters with an arachidic acid internal standard. Cholesterol was measured in total lipid extracts by quantitative GLC after saponification with 0.3 N methanolic NaOH in the presence of a coprostanol internal standard. Total PL and individual PL classes were measured by the ammonium–ferrothiocyanate procedure (37), using a 2:1 mixture of choline PL (CP) and ethanolamine PL (EP) (Sigma Co.) as a standard. EP and CP were submitted to ether-linked lipid analysis following the procedure of Su and Schmidt (38). By using this procedure, alk-1′-enyl-linked lipids are converted into alkyl-substituted dioxanes, and alkyl-linked lipids are converted into alkylglycerols by hydrogenolysis with sodium *bis*-(2-methoxy ethoxy)-aluminum hydride in toluene (Eastman Kodak Company Co., Rochester, NY). Alkyl-substituted dioxanes and alkylglycerols were separated by thin-layer chromatography (TLC) on silica gel H in the solvent system hexane/diethyl ether (40:60, vol/vol), and then measured by quantitative GLC using dimethoxytetradecane and pentadecylglycerol, respectively, as internal standards.

GLC analyses. Methyl ester derivatives of fatty acids from TG, EP, CP, and phosphatidylinositol + phosphatidylserine (PI + PS) were obtained by heating the samples at 80°C for 12 h in a solution of 5% (vol/vol) H_2SO_4 in methanol/benzene (25:1, vol/vol). Fatty acid methyl esters were extracted into hexane, concentrated under a stream of nitrogen and then analyzed isothermally at 185°C on six-foot glass columns packed with EGSS-X on Gas-Chrom P 100-200 mesh (Supelco, Bellefonte, PA). Fatty acid methyl esters were identified by their retention times relative to those of standard mixtures (Supelco). Alkyl-substituted dioxanes and isopropylidene derivatives of alkylglycerols (39) were analyzed at 195°C on six-foot glass columns packed with EGSS-X on Gas-Chrom P 100–200 mesh. Cholesterol was analyzed at 265°C on a six-foot glass column packed with 3% OV 17 on 80–100 mesh Supelcoport (Supelco). Columns were mounted in a Perkin-Elmer gas chromatograph model 3920 (Perkin-Elmer, Norwalk, CT) equipped with flame-ionization detectors. Peak areas were calculated by using a Perkin-Elmer Sigma 10 integrator.

Statistical analysis. Statistical significance of the differences between differentiated and undifferentiated HL-60 cells was determined by Student's *t*-test.

RESULTS AND DISCUSSION

The amounts of total PL, cholesterol, and TG in undifferentiated HL-60 cells are shown in Table 1, and are similar to those found for other transformed cell types (40–42). Differentiation of HL-60 cells into macrophages by TPA was accompanied by a decrease in total PL. This change was also found in HL-60 cells differentiated into granulocytes by DMSO (32) or retinoic acid (33). Moreover, TPA-induced differentiation was accompanied by an increase of TG, a change also reported by Cabot *et al.* (29). An increase of TG content was also reported by Naito *et al.* (32) in HL-60 cells differentiated into granulocytes by retinoic acid.

TPA-induced differentiation was characterized by a significant decrease in CP content, a change also found in HL-60 cells differentiated into granulocytes by retinoic acid (32) or DMSO (33) (Table 2).

Both EP and CP of undifferentiated HL-60 cells contain appreciable amounts of ether-linked subclasses with a prevalence of 1-alk-1′-enyl-2-acyl molecular species in EP (i.e., ethanolamine plasmalogens), and of 1-alkyl-2-acyl molecular species in CP (Table 3). Differentiation of HL-60 cells into macrophages was accompanied by a marked increase of 1-alk-1′-enyl-2-acyl- and 1-alkyl-2-acyl-*sn*-glycero-3-phospho-

TABLE 1
Changes of Lipid Composition in HL-60 Cells Differentiated into Macrophages by TPA[a]

Lipid class	HL-60 cells	TPA-treated cells
Total phospholipids	183.0 ± 14.9	153.0 ± 7.9[b]
Total cholesterol	45.0 ± 2.2	37.0 ± 6.3
Triacylglycerols	6.0 ± 0.3	10.0 ± 0.4[c]
Cholesterol/phospholipid molar ratio	0.251 ± 0.01	0.272 ± 0.01

[a]TPA, 12-O-tetradecanoyl-phorbol-13-acetate. Results are expressed as nmol/10^7 cells and represent the mean value ± SEM of three separate experiments.
[b]$P < 0.05$.
[c]$P < 0.005$.

TABLE 2
Changes of Phospholipid Composition in HL-60 Cells Differentiated by TPA[a]

Phospholipid class[b]	HL-60 cells	TPA-treated cells
CP	104.0 ± 1.5 (56.8)	83.2 ± 1.3 (54.4)[c]
EP	48.3 ± 1.8 (26.4)	41.2 ± 2.8 (26.9)
PI + PS	14.3 ± 0.4 (7.8)	14.1 ± 0.3 (9.2)
SP	13.0 ± 1.4 (7.1)	11.6 ± 0.8 (7.6)
DPG	2.9 ± 0.6 (1.6)	2.6 ± 0.3 (1.7)
PA	0.5 ± 0.0 (0.3)	0.3 ± 0.0 (0.2)

[a]Values are expressed as nmol/10^7 cells and represent the mean value ± SEM of three separate experiments. Numbers in parentheses are percentages of total phospholipids.
[b]CP, choline phospholipids; EP, ethanolamine phospholipids; PI + PS, phosphatidylinositol + phosphatidylserine; SP, sphingomyelin; DPG, diphosphatidylglycerol; PA, phosphatidic acid; other abbreviation as in Table 1.
[c]$P < 0.05$.

ethanolamine, and a decrease of 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. A similar increase in the 1-alk-1′-enyl-2-acyl-subclass of EP was observed on differentiation of HL-60 cells into granulocytes (31–33). On the other hand, the increase of 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine found in our HL-60 cells differentiated into macrophages is in contrast with the decrease of the same subclass reported by Suga *et al.* (33) for HL-60 cells differentiated into granulocytes, a discrepancy possibly related to the different pattern of differentiation in HL-60 cells treated with TPA or DMSO. It is worth noting that the levels of ethanolamine plasmalogens found in HL-60 cells on differentiation into macrophages approximated those reported for peritoneal and alveolar macrophages (44–47). An increase in the level of ethanolamine plasmalogens was also found following DMSO-induced differentiation of N1E-115 neuroblastoma cells (48). Moreover, maturation of human central nervous system was accompanied by an increase of ethanolamine plasmalogens in myelin (49).

As shown in Table 4, 16:0 was the major alk-1′-enyl chain, with smaller amounts of 18:0 and 18:1 alk-1′-enyl chains, in the alk-1′-enyl-acyl subclasses of both EP and CP in undifferentiated HL-60 cells. Similarly, 16:0 was found to be the major alkyl chain in the alkyl-acyl subclass of CP, whereas in the alkyl-acyl subclass of EP there were equivalent levels of 16:0 and 18:0 alkyl chains, and smaller, but substantial, levels of 16:1 and 18:1 alkyl chains. TPA-induced differentiation of HL-60 cells was not accompanied by any significant changes of alk-1′-enyl and alkyl chain compositions of ether-linked EP and CP subclasses.

In undifferentiated HL-60 cells, the fatty acid profiles of EP, CP, and PI + PS were characterized by a high level of oleic acid (18:1n-9) and low percentages of linoleic (18:2n-6), arachidonic (20:4n-6), and 22:polyunsaturated fatty acids (Table 5). These characteristics in fatty acid profiles are similar to those reported for other types of malignant cells (36,41,42,50,51). Differentiation of HL-60 cells into macrophages was accompanied by a significant decrease of palmitoleic (16:0n-9) in CP, but there was no change in the level of oleic acid in the various PL classes. Moreover, the level of arachidonic acid in EP of differentiated HL-60 cells was twice as high as that observed in undifferentiated cells, and reached a value very close to that reported for human alveolar macrophages (52). In contrast, Cabot and Welsh (30) reported no difference in arachidonic acid level of the total PL in HL-60 cells differentiated into macrophages. A marked increase in the level of arachidonic acid also was found in individual PL classes of HL-60 cells on differentiation into granulocytes (32), as well as in EP of N1E-115 neuroblastoma cells induced to differentiate by DMSO treatment (48). The increase in the proportion of arachidonic acid in EP of our HL-60 cells differentiated into macrophages corresponds to the rise of the ethanolamine plasmalogen level in these cells. Similarly, arachidonic acid is mainly present in the alk-1′-enyl-acyl subclass of EP in resident alveolar macrophages (45), thioglycollate-elicited peritoneal macrophages (47,53), human neutrophils (54,55), and HL-60 cells following differentiation into granulocytes (32).

In conclusion, differentiation of HL-60 cells into macrophage-like cells, caused by TPA treatment, was associated with an increase in the level of ethanolamine plasmalogens and an enrichment of EP in arachidonic acid. It is difficult to ascertain the biological relevance of this change in the macrophagic differentiation of HL-60 cells, due to our poor knowledge of the physiological function of ethanolamine plasmalogens (56). However, the recent observation that the characteristic vinyl-ether linkage of ethanolamine plasmalogens protects animal cell membranes from certain oxidative stresses (57) points to the possibility that elevated levels of this lipid may permit macrophages to scavenge reactive oxygen species produced during macrophage activation. Moreover, the high level of ethanolamine plasmalogens in HL-60 cells on differentiation into macrophages may reflect a requirement for these particular phospholipid molecules in the generation of bioactive lipids, including PAF and eicosanoids, which are important in macrophage function (55,58–60). Indeed, ethanolamine plasmalogens are the major source of arachidonic acid precursor for eicosanoid production in granulocytes (55,61). This release of arachidonic acid generates 1-alk-1′-enyl-2-lyso-*sn*-glycero-3-phosphoethanolamine, which acts as an acceptor of arachidonic acid released from 1-alkyl-2-acyl-*sn*-

TABLE 3
Changes of 1-Alk-1′-Enyl-2-Acyl, 1-Alkyl-2-Acyl, and 1,2-Diacyl Subclasses of EP and CP in HL-60 Cells Differentiated into Macrophages by TPA[a]

	EP		CP	
Subclasses	HL-60 cells	TPA-treated cells	HL-60 cells	TPA-treated cells
1-Alk-1′-enyl-2-acyl	35.4 ± 1.6	62.6 ± 1.5[b]	6.0 ± 1.0	7.7 ± 0.4
1-Alkyl-2-acyl	3.2 ± 0.4	6.7 ± 0.3[c]	14.8 ± 1.3	10.6 ± 0.8[d]
1,2-Diacyl	61.4 ± 1.2	30.4 ± 1.2[d]	79.2 ± 1.8	81.6 ± 1.2

[a]Values were calculated as described by Fallani *et al.* (Ref. 43), and are expressed as percentages of total EP and CP in each cell type. They are expressed as a mean value ± SEM for three separate experiments. Abbreviations as in Tables 1 and 2.
[b]$P < 0.01$.
[c]$P < 0.05$.
[d]$P < 0.005$.

TABLE 4
Alkyl and Alk-1′-Enyl Chains of Ether-Linked Subclasses of EP and CP in Undifferentiated HL-60 and TPA-Treated HL-60 Cells[a]

	EP		CP	
Hydrocarbon chain	HL-60 cells	TPA-treated cells	HL-60 cells	TPA-treated cells
Alk-1′-enyl				
16:0	67.9 ± 3.9	57.1 ± 5.1	57.0 ± 1.6	54.4 ± 5.4
16:1	ND[b]	ND	ND	ND
18:0	21.4 ± 3.1	28.9 ± 7.2	33.8 ± 4.3	39.9 ± 8.7
18:1	10.7 ± 1.0	13.1 ± 2.6	9.2 ± 3.1	6.5 ± 1.6
Alkyl				
16:0	30.9 ± 7.4	33.8 ± 1.6	79.0 ± 2.5	81.4 ± 1.3
16:1	18.0 ± 4.7	27.2 ± 2.3	2.8 ± 1.1	2.3 ± 0.3
18:0	39.6 ± 7.3	24.4 ± 1.1	11.2 ± 3.0	7.6 ± 1.1
18:1	11.5 ± 0.8	14.6 ± 6.1	7.0 ± 1.1	8.7 ± 2.6

[a]Values are percentages by weight of total alkyl or alk-1′-enyl chains in each subclass and represent mean values ± SEM of three separate experiments. Abbreviations as in Tables 1 and 2.
[b]Not detectable.

TABLE 5
Changes in the Fatty Acid Composition of EP, CP, and PI + PS in HL-60 Cells Differentiated into Macrophages by TPA[a]

	EP		CP		PI + PS	
Fatty acid	HL-60 cells	TPA-treated cells	HL-60 cells	TPA-treated cells	HL-60 cells	TPA-treated cells
16:0	32.7 ± 7.6	30.4 ± 4.1	35.3 ± 4.2	41.0 ± 0.5	12.0 ± 1.8	15.6 ± 1.4
16:1n-9	6.7 ± 2.3	5.5 ± 2.6	12.4 ± 1.7	6.7 ± 0.3[b]	6.0 ± 1.0	3.4 ± 0.3
18:0	13.4 ± 3.4	18.4 ± 1.3	5.6 ± 1.0	6.1 ± 0.5	39.0 ± 0.2	42.8 ± 2.0
18:1n-9	36.5 ± 8.6	30.5 ± 3.7	39.9 ± 2.4	40.3 ± 0.3	30.5 ± 3.2	28.2 ± 2.1
18:2n-6	0.5 ± 0.0	0.3 ± 0.3	trace	0.8 ± 0.1	0.1 ± 0.0	1.2 ± 0.5
20:4n-6	4.3 ± 0.3	10.1 ± 0.5[b]	1.1 ± 0.5	1.3 ± 0.1	2.4 ± 0.0	3.3 ± 0.6
22:5n-3	2.2 ± 0.7	1.6 ± 0.5	0.6 ± 0.2	1.1 ± 0.2	1.1 ± 0.5	0.9 ± 0.2
22:6n-3	1.7 ± 1.0	1.1 ± 0.5	3.6 ± 1.0	1.6 ± 0.2	3.0 ± 1.0	2.2 ± 0.8
Others	2.0 ± 0.9	2.0 ± 0.3	1.5 ± 0.1	1.1 ± 0.2	5.9 ± 0.5	2.4 ± 0.5

[a]Values are percentage by weight of total fatty acids in each phospholipid and represent mean values ± SEM of three separate experiments. Abbreviations as in Tables 1 and 2.
[b]$P < 0.01$.

glycero-3-phosphocholine during the transacylation reaction, which is a critical event in PAF biosynthesis (58–60). Ethanolamine plasmalogens also can be converted into an ethanolamine plasmalogen analog of PAF, as has been reported by Snyder *et al.* (60) in HL-60 cells following differentiation into granulocytes.

ACKNOWLEDGMENTS

This work has been supported by grants from the Consiglio Nazionale delle Ricerche (Special Project: ACRO), from the Associazione Italiana della Ricerca sul Cancro (AIRC), and from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, 40–60%). We thank Prof. A. Fonnesu, Chairman of the Institute of General Pathology, University of Florence, for his interest in this study. We also gratefully acknowledge the generous help received by Dr. Valeria Santini, of Department of Hematology, University of Florence, for the assay of the differentiation markers. We express our deepest gratitude to Dr. Nuria Suesa, of the Menarini Laboratorios (Barcelona, Spain), for providing the HL-60 cells used in this study.

REFERENCES

1. Papahadjopoulos, D., Cowden, M., and Kimelberg, H. (1973) *Biochim. Biophys Acta 330,* 8–26.
2. Deuticke, B., and Ruska, C. (1976) *Biochim. Biophys. Acta 433,* 638–653.

3. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
4. Rethy, A., Tomasi, V., Trevisani, A., and Bernabei, O. (1972) *Biochim. Biophys. Acta 290*, 58–69.
5. Hebdon, G.M., Levine III, H., Sahyoun, N.E., Schmitges, C.J., and Cuatrecasas, P. (1981) *Proc. Natl. Acad. Sci. USA 78*, 120–123.
6. Brivio-Haugland, R.P., Louis, S.L., Musch, K., Waldeck, N., and Williaams, M.A. (1976) *Biochim. Biophys. Acta 433*, 150–163.
7. Ross, E.M. (1982) *J. Biol. Chem. 257*, 10751–10758.
8. Chambaz, J., Pepin, D.. Robert, A., Wolf, C., and Bereziat, G. (1983) *Biochim. Biophys. Acta 727*, 313–326.
9. Calorini, L., Mugnai, G., Mannini, A., and Ruggieri, S. (1993) *Lipids* 28, 727–730.
10. Solomonson, L.P., Liepknals, V.A., and Spector, A.A. (1976) *Biochemistry 24*, 892–897.
11. Szamel, M., and Resch, K. (1981) *J. Biol. Chem. 256*, 11618–11623.
12. Ginsberg, B.H., Brown, T.J., Simon, S., and Spector, A.A. (1981) *Diabetes 30*, 773–780.
13. Ginsberg, B.H., Jabour, J., and Spector, A.A. (1982) *Biochim. Biophys. Acta 690*, 157–164.
14. Horwitz, A.F., Hatten, M.E., and Burger, M.M. (1974) *Proc. Natl. Acad. Sci. USA 71*, 3115–3119.
15. Tombaccini, D., Ruggieri, S., Fallani, A., and Mugnai, G. (1980) *Biochem. Biophys. Res. Comm. 96*, 1109–1115.
16. Curtis, A.S.G., Chandler, C. and Picton, M. (1975) *J. Cell. Sci. 18*, 375–384.
17. Van Golde, L.M.G. (1986) in *Lipids and Membranes. Past, Present and Future* (Op Den Kamp, J.A.F., Roelofsen, B., and Wirtz, K.W.A., eds.) pp. 287–306, Elsevier, Amsterdam, Oxford, New York.
18. Neelands, P.J., and Clandinin, M.T. (1983) *Biochem. J. 212*, 573–583.
19. Alam, S.Q., Ren, Y-F., and Alam, B.S. (1989) *Lipids 24*, 39–44.
20. Krall, J.F., Leshon, S.C., and Korenman, S.G. (1985) *J. Biol. Chem. 260*, 9685–9691.
21. Orly, J., and Schramm, M. (1975) *Proc. Natl. Acad. Sci. USA 72*, 3433–3437.
22. Bakardjieva, A., Galla, H.J., and Helmreich, E.J.M. (1979) *Biochemistry 18*, 3016–3023.
23. Friend, C., Scher, W., Holland, J.G., and Sato, T. (1971) *Proc. Natl. Acad. Sci. USA 68*, 378–382.
24. Sachs, L. (1978) *Nature 274*, 535–539.
25. Collins, S.J. (1987) *Blood 70*, 1233–1243.
26. Rovera, G., Santoli, D., and Damsky, C. (1979) *Proc. Natl. Acad. Sci. USA 76*, 2779–2783.
27. Camussi, G., Bussolino, F., Ghezzo, F., and Pegoraro, L. (1982) *Blood, 59*, 16–22.
28. Ziboh, V.A., Wong, T., Wu, M.C., and Yunis, A.A. (1986) *J. Lab. Clin. Med. 108*, 161–166.
29. Cabot, M.C., Welsh, C., Callaham, F., and Huberman, E. (1980) *Cancer Res. 40*, 3674–3679.
30. Cabot, M.C., and Welsh, C. (1981) *Cancer Res. 41*, 4910–4915.
31. Billah, M.M., Eckel, S., Myers, R.F., and Siegel, M.I. (1986) *J. Biol. Chem. 261*, 5824–5831.
32. Naito, M., Kudo, I., Nakagawa, Y., Waku, K., Nojiri, H., Saito, M., and Inoue, K. (1987) *J. Biochem. 102*, 155–162.
33. Suga, K., Kawasaki, T., Blank, M.L., and Snyder, F. (1990) *J. Biol. Chem. 265*, 12363–12371.
34. Yam, L.T., Li, C.Y., and Crosby, W.H. (1971) *Am. J. Clin. Pathol.* 55, 283–290.
35. Folch, J., Lees, M., and Sloane-Stanley, G.M. (1957) *J. Biol. Chem. 226*, 497–509.
36. Calorini, L., Fallani, A., Tombaccini, D., Mugnai, G., and Ruggieri, S. (1987) *Lipids* 22, 651–656.
37. Steward, J.C.M. (1980) *Anal. Biochem. 104*, 10–14.
38. Su, K.L., and Schmidt, H.H.O. (1974) *Lipids 9*, 208–213.
39. Wood, R. (1967) *Lipids 2*, 199–203.
40. Chao, F.C., Eng, L.F., and Griffin, A. (1972) *Biochim. Biophys. Acta 260*, 197–202.
41. Ruggieri, S., Roblin, R., and Black, P.H. (1979) *J. Lipid Res. 20*, 772–783.
42. Calorini, L., Fallani, A., Tombaccini, D., Barletta, E., Mugnai, G., Di Renzo, M.F., Comoglio, M.P. and Ruggieri, S. (1989) *Lipids 24*, 685–690.
43. Paltauf, F. (1983) in *Ether Lipids—Biochemical and Biomedical Aspects* (Mangold, H.K., and Paltauf, F., eds.) pp. 309–353, Academic Press, New York.
44. Fallani, A., Bracco, M., Tombaccini, D., Mugnai, G., and Ruggieri, S. (1982) *Biochim. Biophys. Acta 711*, 208–212.
45. Sugiura, T., Onuma, Y., Sekigushi, N., and Waku, K. (1982) *Biochim. Biophys. Acta 712*, 515–522.
46. Sugiura, T., Nakajima, M., Sekiguchi, N., Nakagawa, Y., and Waku, K. (1983) *Lipids 18*, 125–129.
47. Nakagawa, Y, Sugiura, T., and Waku, K. (1985) *Biochim. Biophys Acta 833*, 323–329.
48. Chapkin, R.S., and Carmichael, S.L. (1990) *J. Nutr. 120*, 825–830.
49. Murphy, E.J., and Horrocks, L.A. (1993) *Biochim. Biophys. Acta 1167*, 131–136.
50. Fishman, M.A., Agrawal, H.C., Alexander, A., and Golterman, J. (1975) *J. Neurochem. 24*, 689–694.
51. Montaudon, D., Louis, J.L., and Robert, J. (1981) *Lipids 16*, 293–297.
52. Fallani, A., Arcangeli, A., and Ruggieri, S. (1988) *Biochem. J. 255*, 731–736.
53. Sahu, S., and Lynn, W.S. (1977) *Inflammation 2*, 83–91.
54. Akoh, C.C., and Chapkin, R.S. (1990) *Lipids 25*, 613–617.
55. Mueller, H.W., O'Flaherty, J., Greene, D.G., Samuel, M.P., and Wykle, R.L. (1984) *J. Lipid Res. 25*, 383–388.
56. Chilton, F.H., and Connel, T.R. (1988) *J. Biol. Chem. 263*, 5260–5265.
57. Zoeller, R.A., Morand, O.H., and Raetz, C.R.H. (1988) *J. Biol. Chem. 23*, 11590–11596.
58. Uemura, Y., Lee, T., and Snyder, F. (1991) *J. Biol. Chem. 266*, 8268–8272.
59. Nieto, M.L., Venable, M.E., Bauldry, S.A., Greene, D.G., Kennedy, M., Bass, D.A., and Wykle, R.L. (1991) *J. Biol. Chem. 266*, 18699–18706.
60. Snyder, F., Uemura, Y., Lee T-c., and Blank, M.L. (1993) in *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury* (Nigam, S., Narnett, L.J., Honn, K.V., and Walden L.J., eds) pp. 691–697, Kluwer Academic Publishers, Boston.
61. Tessner, T.G., Greene, D.G., and Wykle, R.L. (1990) *J. Biol. Chem. 265*, 21032–21038.

[Received February 20, 1995, and in final revised form June 23, 1995; Revision accepted July 3, 1995]

Pathogenesis of Oleic Acid-Induced Lung Injury in the Rat: Distribution of Oleic Acid During Injury and Early Endothelial Cell Changes

Greg Beilman*

Departments of Surgery and Internal Medicine, University of Kansas School of Medicine-Wichita, Wichita, Kansas 67214

ABSTRACT: Oleic acid-induced lung injury, a well-described laboratory model for acute pulmonary injury in the rat and other species, causes morphologic and cellular changes similar to human adult respiratory distress syndrome (ARDS). Experiments were performed to test the hypothesis that the initial event of oleic acid lung injury is damage of the pulmonary vascular endothelium by oleic acid, with subsequent pulmonary damage and inflammation. Oleic acid levels were followed in the lung and other tissues by measuring accumulation of ^{14}C-oleic acid; the direct effects of oleic acid and other fatty acids on rat endothelial cells, alveolar type II cells, and hepatocytes in culture were determined. Lung tissue from treated rats was also examined by light and electron microscopy for evidence of endothelial cell damage. At 30 min after injury, oleic acid reached high concentrations in lung tissue as demonstrated by presence of radiolabel (3.24×10^{-6} moles per gram of tissue), with counts in the lung nearly an order of magnitude greater than in any other organ measured. Oleic acid was present in the lung mostly as free fatty acid (85%), and was also present in the alveolar fluid supernatants, rather than being cell-associated (1.7×10^{-7} moles vs. 1.1×10^{-8} moles at 30 min). Oleic acid was toxic to endothelial cells after one minute of exposure at concentrations of 5×10^{-4} M and above. Electron microscopy showed endothelial cell changes as early as 10 min after induction of injury *in vivo*, including the presence of endothelial cell blebbing. The results of these studies suggest that the initial event in oleic acid lung injury is damage to the pulmonary vascular endothelial cell.
Lipids 30, 817–823 (1995).

Adult respiratory distress syndrome (ARDS) afflicts 150,000 patients yearly in the United States, with a mortality rate of over 50%. This rate has not improved since ARDS was first described in 1967 despite the use of positive-pressure ventilation and other therapies (1). The predisposing conditions associated with ARDS are quite diverse, ranging from endotoxic shock to cardiopulmonary bypass. A variety of inflammatory mediators have been investigated in both human ARDS and animal models of acute lung injury, but blocking the effects of any one of these mediators has met with only limited success and no improvement in outcome. This suggests that ARDS is likely to be the final result of several different pathways of pulmonary injury.

Oleic acid (OA), an 18-carbon unsaturated fatty acid, causes morphologic and cellular changes similar to human ARDS when given intravenously (2–6), as recently reviewed (7). It is possible that this injury is similar to that caused in humans by fat embolus, pancreatitis, and possibly narcotic overdose; however, there are scant data on the initial events that occur during acute lung injury from OA. It seems unlikely that complement plays a significant role in OA lung injury, since levels of complement have not been noted to change during its early stages, and depletion of the third component of complement by cobra venom toxin failed to abrogate the injury (2). Similarly, depletion of platelets has failed to significantly affect the course of this form of lung injury (2,8). Although depletion of neutrophils in one set of experiments resulted in decreased permeability of the lung in rats after OA lung injury, such depletion did not lead to differences in histology or bronchoalveolar lavage (BAL) findings (9). Oxygen radicals have been found to be important in the etiology of certain types of pulmonary injury (10–12), but several studies examining oxygen radicals in OA lung injury have argued against a role of this mediator (9,13,14). This suggests that there is an early component of OA lung injury not related to any of the above factors.

The hypothesis that the initial event of OA lung injury is damage of the pulmonary vascular endothelium by OA with subsequent pulmonary damage and inflammation was tested by several approaches. OA levels were measured in the lung and other body tissues after ^{14}C-OA injection to determine relative concentrations of OA in free and esterified forms during early lung injury. The cytotoxic effects of OA and other fatty acids at different concentrations and time intervals were determined with rat endothelial cells and other cells *in vitro*. Tissue samples of lung from rats treated with OA were examined for evidence of endothelial cell damage by electron microscopy.

*Address correspondence at UMHC Box 11, Department of Surgery, University of Minnesota, Minneapolis, MN 55455.

Abbreviations: ARDS, Adult respiratory distress syndrome; ATII, alveolar type II cells; BAL, bronchoalveolar lavage; FBS, fetal bovine serum; HBSS, Hanks' Balanced Salt Solution; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OA, oleic acid; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor-α.

Copyright © 1995 by AOCS Press

EXPERIMENTAL PROCEDURES

Animals. Male Sprague-Dawley rats, free of respiratory disease and weighing 250–350 g, were used in this study (Sasco Labs, Omaha, NE). All rats were fed and watered *ad libitum* and housed in microisolator cages. All protocols were approved by the Animal Use Committees of the University of Kansas School of Medicine–Wichita and Veterans Affairs Medical Center–Wichita.

Reagents. Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), and RPMI-1640 were obtained from JRH Bioscience (Lenexa, KS). ^{14}C-labeled OA was obtained from New England Nuclear (Boston, MA). Oleic, linolenic, γ-linolenic, linolenaidic, 14-*cis*-eicosenoic, and arachidonic acids and bovine serum albumin were obtained from Sigma (St. Louis, MO).

Cell culture and harvesting. Endothelial cells were isolated from rat pulmonary arteries using the methods of Habliston *et al.* (15) and were grown in complete media consisting of RPMI-1640 with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 10% Nu-Serum (Collaborative Research, Cambridge, MA), heparin 20 μg/mL (Fisher, St. Louis, MO), and retinal-derived growth factor (20 μL/mL) (16). Cells were identified as endothelial by morphology and by the ability to take up DiI-ac-LDL, low-density lipoprotein labeled with the fluorescent probe, DiI (Biomedical Technologies Inc., Stoughton, MA). Cells were passaged with trypsin onto gelatin-coated plates. Cells at passages 6–12 were used for this series of experiments.

Rat hepatocytes were obtained using collagenase harvesting as previously described (17) and plated onto 96-well plates coated with bovine dermal collagen (Celtrix, Santa Clara, CA) at a concentration of 4×10^4 cells/well. Cells were used 48–72 h after harvest.

Rat alveolar type II cells (ATII) were harvested using the methods of Dobbs *et al.* (18). ATII morphology was confirmed with electron microscopy and Papinicolou stain demonstrating >90% ATII cells. These cells were plated on 96-well plates at 4×10^4 cells/well and were allowed to adhere for 48 h prior to use.

Induction of lung injury. Lung injury was induced under ketamine anesthesia as previously described (2). Rats under ketamine anesthesia received OA suspended in a solution of bovine serum albumin dissolved in PBS (1 mg/mL) given *via* femoral vein cut down. For experiments using radiolabeled OA, 10 uCi of ^{14}C-labeled OA were added to the dose received by each animal. The total dose of exogenous OA administered per animal was 8.5×10^{-5} moles.

Animal protocol. Rats were sacrificed at 30 min, 2 h, 4 h, and 12 h (n = 3 for each group) after the administration of OA. Animals were anesthetized; then liver, spleen, and kidneys were removed, and blood was drawn into heparinized syringes. BAL was performed using five aliquots of HBSS at 4°C (10 cc/aliquot). The chest cavity was then opened, and lungs, thymus, and heart were removed. The brain was removed after opening the cranium. Urine was aspirated from the bladder at the time of sacrifice. Blood and BAL fluids were centrifuged, and supernatants and cell pellets were stored separately. All samples were weighed and stored at −70°C until assayed.

Measurement of OA levels. A small sample (100–200 mg) was taken from each organ, weighed, homogenized, and then dissolved in Scintigest tissue solubilizer (Fisher) (1.5 mL/100 mg tissue) and water (200 μL/100 mg tissue). This mixture was then placed in scintillation cocktail (Scintiverse, Fisher). Constant volumes of BAL fluid, cells, plasma, and red blood cell pellet were added to scintillation cocktail. Samples were read on a scintillation counter with window set for ^{14}C. Quenching was evaluated for each tissue by adding a known amount of ^{14}C OA to organs prepared from an uninjected animal in the same fashion as noted above. With the exception of the red blood cell prep (necessitating a dilution of 1:4000), no significant quenching was noted.

Data were collected as cpm. By determining the number of cpm in each organ [(total organ weight/weight of counted sample) × cpm of sample] and by dividing this number by the total cpm injected into the animal, the distribution of counts in each organ at each time interval was determined. An approximation of the concentration of exogenous OA in selected instances was determined by multiplying this percentage by the total number of moles injected in each animal (8.53×10^{-5} moles). This calculation does not take into account endogenous sources of OA.

In order to determine the lipid distribution of the radiolabel, tissue from the lung, liver, kidney, and blood from a rat at each time period was extracted with chloroform/methanol and was dried. This extract was diluted with chloroform and spotted onto thin-layer chromatography plates and run using petroleum ether/diethyl ether/glacial acetic acid solvent (80:20:1). Ultraviolet light was used to identify cholesterol ester, triglyceride, free fatty acid, and phospholipid bands which were scraped into vials with scintillation cocktail and counted. The band remaining at the origin was assumed to be the phospholipid fraction. This technique does not measure potential radiolabeled water-soluble components that may be present at the later time intervals.

In vitro *experiments.* Endothelial cells were plated on 96-well tissue culture plates and were allowed to grow to confluence. Solubilized OA was prepared by dissolving OA (10 μL) in 150 μL ethanol and by adding 750 μL of distilled water to this solution. NaOH (1N) was added in a dropwise fashion until OA was completely solubilized (13). Other fatty acids were prepared in the same fashion. Dilutions of these solutions in complete media were filter-sterilized. Diluent without fatty acids was not noted to exhibit cytotoxicity at concentrations used in these experiments. In the first series of experiments, the time course of OA cytotoxicity was evaluated. OA, at a concentration of 5×10^{-4}M and 10^{-3}M in complete media, was added to endothelial cells, grown to confluence and OA-induced cytotoxicity was measured at intervals using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to assess viable cells (19). In this assay,

viable cells convert MTT to an insoluble formazan product, which is measured by determining the absorbance of each sample at 560 nm after solubilizing the formazan product in isopropanol.

In another experiment, the dose response of OA was assessed. Dilutions of OA in complete media were added to endothelial cells and incubated for 4 h at 37°C. Control wells contained either media alone or 3M guanidine HCl (Sigma). MTT assay was then performed to determine cytotoxicity. These experiments were repeated four times to ensure consistency. The percentage of cytotoxicity for each concentration of OA was calculated using the formula:

$$\% \text{ cytotoxicity} = 1 - \left[\frac{A_{\text{experimental}} - A_{\text{guanHCl}}}{A_{\text{media}} - A_{\text{guanHCl}}}\right] \times 100 \qquad [1]$$

where $A_{\text{experimental}}$ equals the absorbance at 560 nm of cells treated with OA, A_{guanHCl} equals the absorbance at 560 nm of cells treated with guanidine HCl, and A_{media} equals the absorbance at 560 nm of cells treated with media. These experiments were then repeated with linolenic, γ-linolenic, linolenaidic, 14-*cis*-eicosenoic, and arachidonic acids, using different concentrations of serum with OA. The effects of OA were compared in different rat primary cell cultures. Rat endothelial cells, hepatocytes, and ATII cells were treated for 4 h with OA at various concentrations, followed by MTT assay to assess cytotoxicity.

Electron microscopy. Rats with OA lung injury were sacrificed at 10 min, 30 min, and 1, 2, 4, and 8 h after induction of injury. A control animal injected with carrier solution only was sacrificed as well. The lungs were immediately removed, and tissue was diced into small sections. Biopsies of lung tissue for electron microscopy were taken from areas bordering on grossly hemorrhagic areas of the lungs and were fixed in cold 3% phosphate-buffered glutaraldehyde. The tissue was post-fixed in 2% phosphate buffered OsO_4 (20), dehydrated in graded ethanol, and was embedded in Epon/Araldite (21). Blocks were thick sectioned (2–3 microns; LKB microtome, Bromma, Sweden) and were stained with paragon (22) for orientation. Ultra-thin sections (50–60 nm) were stained with uranyl acetate and lead citrate (23,24). Thin sections were examined, and electron photomicrographs were taken, using a Philips 201 transmission electron microscope (Einthoven, Holland).

Additional tissue specimens were fixed in formaldehyde and were stained with hematoxylin and eosin for routine light microscopy.

Statistical analysis. Means and standard deviations were determined for data, and appropriate data points were compared using Student's unpaired *t*-test with Bonferroni's adjustment for multiple comparisons.

RESULTS

Counts were highest in the lungs 30 min after induction of lung injury. If the assumption is made that most OA is not initially metabolized, this yields a concentration of exogenous OA of 3.24×10^{-6} moles per gram of tissue. These counts are nearly an order of magnitude greater than in any other organ (Table 1). This trend did not reach significance because of small group numbers. Counts were also present in BAL fluid of rats after experimental lung injury, primarily in the BAL supernatants, rather than cell-associated ($4.2 \pm 1.9 \times 10^4$ cpm vs. $3 \pm 3 \times 10^3$ cpm at 30 min). There was only a 22% decrease in counts over the 12-h period of the experiment. Counts were found in other organs in varying amounts, with the highest levels (after the lung) in the heart and liver (Table 1). The approximate half-life of counts, presumably from OA, in the lung parenchyma, was 2½ to 3 h by extrapolation.

TABLE 1
Radiolabel in Various Tissue Compartments[a]

Compartment	Thirty minutes	Two hours	Four hours	Twelve hours
Lung	806 ± 467	297 ± 149	276 ± 138	102 ± 21
Heart	161 ± 99	57 ± 21	34 ± 11	15 ± 3
Liver	159 ± 83	89 ± 23	55 ± 13	25 ± 2
Kidney	53 ± 18	42 ± 11	45 ± 15	28 ± 2
Plasma	34 ± 6	16 ± 5	13 ± 3	34 ± 25
Spleen	32 ± 4	34 ± 11	34 ± 11	21 ± 2
Thymus	23 ± 11	25 ± 8	34 ± 15	23 ± 2
Urine	21 ± 3	49 ± 44	34 ± 11	14 ± 2
Brain	7 ± 4	7 ± 2	8 ± 3	7 ± 1
BAL fluid	42 ± 19	42 ± 13	30 ± 11	33 ± 5
BAL cells	3 ± 3	2 ± 1	3 ± 2	15 ± 1

[a]Data are presented as mean ± SD from three animals at each time period. Data are expressed as cpm per gram of tissue ($\times 10^3$), except for bronchoalveolar lavage (BAL) fluid and cells where data are expressed as cpm $\times$ 10^3 in total collected specimen. The cpm per gram of tissue are increased in pulmonary tissues as compared with other compartments with $P < 0.1$ (Student's unpaired *t*-test).

At 30 min after lung injury, 85% of radioactivity was noted to be associated with the free fatty acid fraction of extracted lipids (Fig. 1). This percentage decreased in a time-dependent manner over the next 12 h to 28%, while the phospholipid

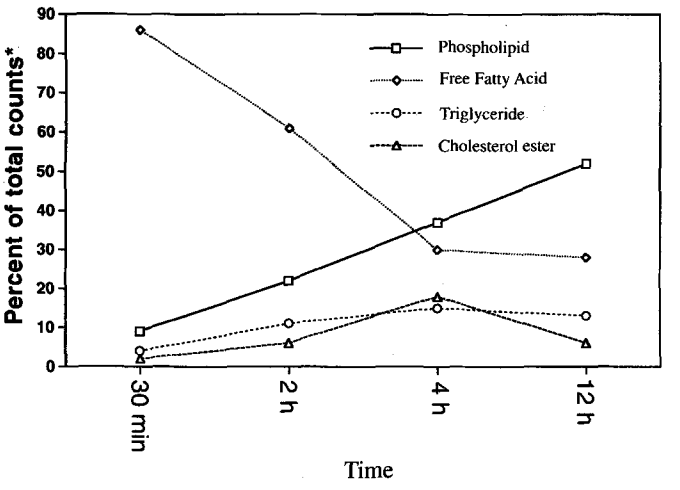


FIG. 1. Percent of total cpm of each fatty acid fraction separated by thin-layer chromatography from rat lungs 30 min, 2 h, 4 h, and 12 h after oleic acid lung injury. *Total cpm at each time point in the fatty acid fraction of the lung were: 30 min, 5142; 2 h, 2134; 4 h, 1252; and 12 h, 3529.

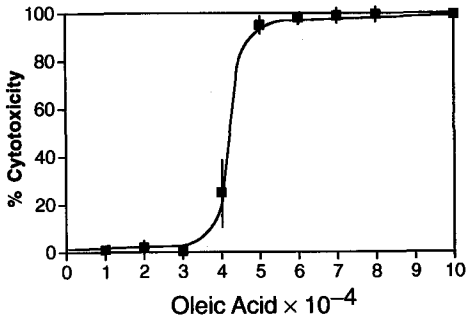


FIG. 2. Toxicity of oleic acid on endothelial cell subcultures. Cell viability was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay after 4 h of culture with oleic acid at varied concentrations (37°C, 5% CO_2). Error bars denote standard deviation.

fraction increased over this time period. In the liver and the kidney, the greatest proportion of radiolabel was in the triglyceride fraction at 30 min (40 and 80%, respectively), with the phospholipid fraction again increasing over the 12 h (39 and 33%, respectively).

OA was found to be toxic to rat pulmonary endothelial cells in culture above levels of 6×10^{-4} M (6×10^{-7} moles/mL) when cultured for 4 h. The LD_{50} of OA on endothelial cells after 4 h in culture was approximately 5×10^{-4} M (Fig. 2). There was also significant cytotoxicity to endothelial cells almost immediately upon exposure (59% cytotoxicity at 1 min of exposure at levels of 10^{-3} M). Cytotoxicity increased linearly over time at OA concentrations of 10^{-3} M and 5×10^{-4} M to 90 and 83%, respectively, for cells exposed to OA for 4 h (Fig. 3). This toxicity was found to be significantly decreased with increasing concentrations of serum, with a 4-h exposure of 4×10^{-4} M OA causing 78% cytotoxicity in the presence of 1% FBS, and no toxicity in the presence of 20% FBS (Fig. 4). Different cell types exhibited variable sensitivity to OA as well, with hepatocytes demonstrating no toxicity with concentrations up to 2×10^{-3} M and ATII cells having sensitivity similar to endothelial cells (data not shown). The sensitivity of endothelial cells to a number of fatty acids was tested (Fig. 5). Endothelial cells were most sensitive to γ-linolenic acid (18:3n-6) and least sensitive to arachidonic acid (20:4n-6).

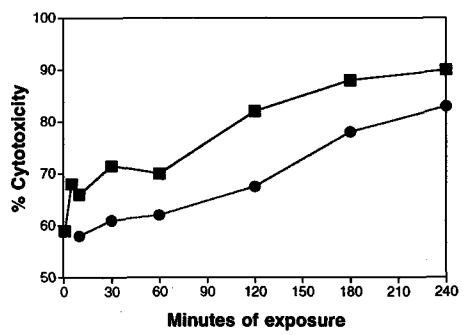


FIG. 3. Time course of cytotoxicity of oleic acid on endothelial cell subcultures. Endothelial cells were cultured in the presence of oleic acid (squares: 10^{-3}M, circles: 5×10^{-4}M) and cytotoxicity determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay.

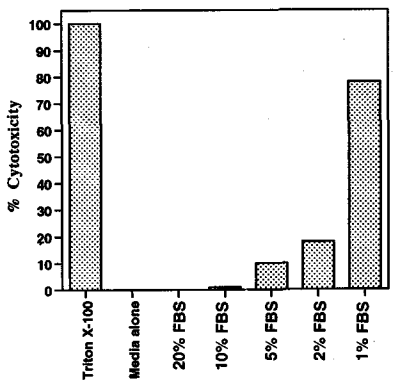


FIG. 4. Serum concentration and endothelial cell injury. Endothelial cells were cultured for 4 h in the presence of oleic acid (5×10^{-4} M) (Triton X-100; Sigma, St. Louis, MO) and different concentrations of fetal bovine serum (FBS). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay performed to determine cytotoxicity. Experiment performed in triplicate with representative experiment shown.

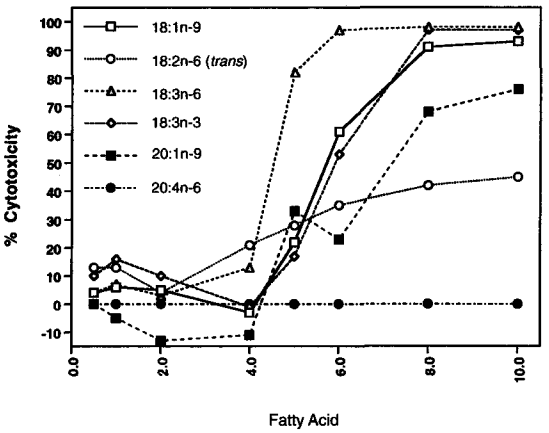
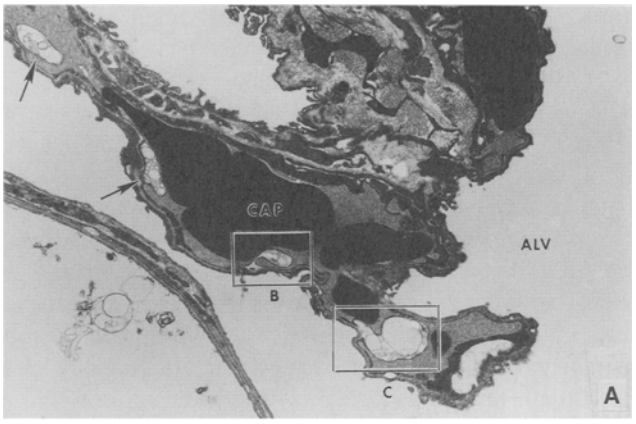
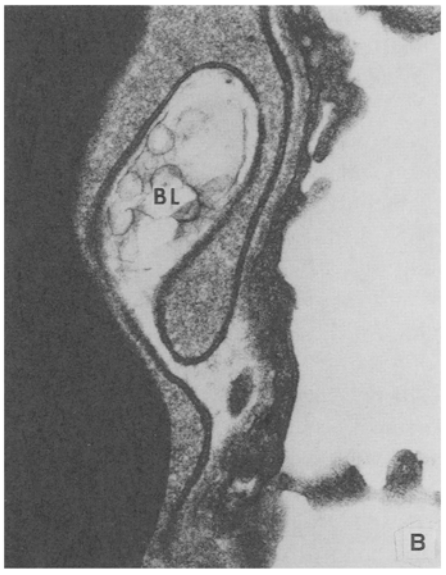


FIG. 5. Cytotoxic effects of different fatty acids on endothelial cells. Endothelial cells were stimulated for 4 h on 96-well plates with various concentrations of fatty acids followed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Experiment performed in triplicate with representative experiment shown.

Gross examination of lungs at the time of sacrifice revealed a patchy distribution of hemorrhagic areas involving the surfaces of both lungs. Light microscopy confirmed this patchy distribution, showing a lipoid necrosis with hemorrhage in the areas of gross involvement, even as early as 10 min after injection of OA.

Electron microscopy performed on grossly unaffected lung tissue from rats with experimental OA lung injury demonstrate definite endothelial cell changes as early as 10 min after induction of injury. These changes are similar to those of endothelial cell blebbing (Fig. 6). Blebbing, a separation of the endothelial membrane from the underlying basement membrane, is a finding strongly suggestive of endothelial cell injury (22). At 10 and 30 min post-injury, the alveolar epithelium was relatively spared in areas examined. Also noted at 10 min post-injury were red cells surrounding amorphous ma-





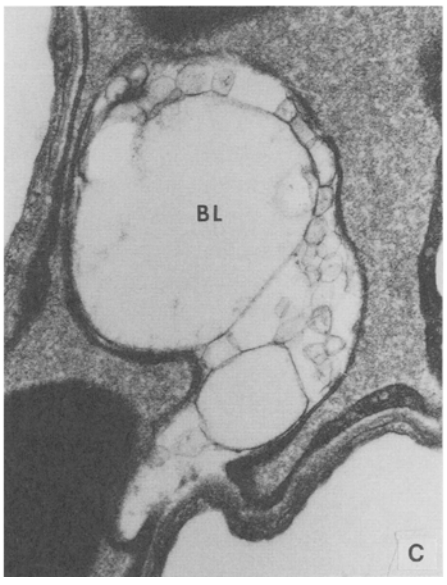


FIG. 6. Transmission electron microscopy of rat lung 10 min after injection of oleic acid. Lung sections were stained with uranyl acetate and lead citrate: A—section including pulmonary capillary (magnification X: 1,600), demonstrating changes consistent with endothelial blebbing. Alveolar space (ALV), capillary space (CAP)—arrows indicate areas suspicious for endothelial blebbing, as seen in areas outlined in A. Higher magnifications seen in B (X: 7,300) and C (X: 9,100) illustrate continuity with the endothelial membrane; BL, bleb.

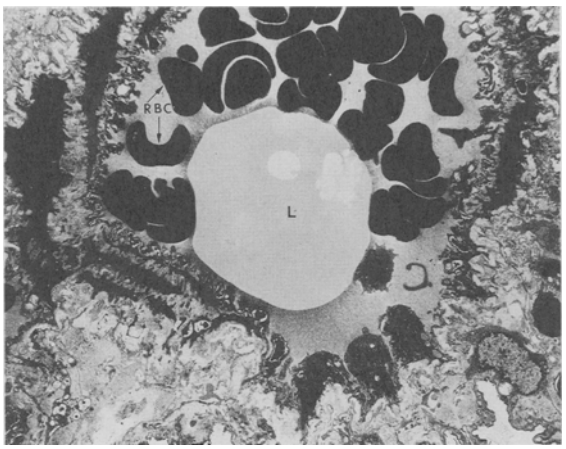


FIG. 7. Transmission electron microscopy of rat lung 10 min after injection of oleic acid, stained with uranyl acetate and lead citrate. Section through small vessel showing red blood cells (RBC, arrows) surrounding material having the appearance of lipid (L), possibly an oleic acid droplet (magnification X: 700).

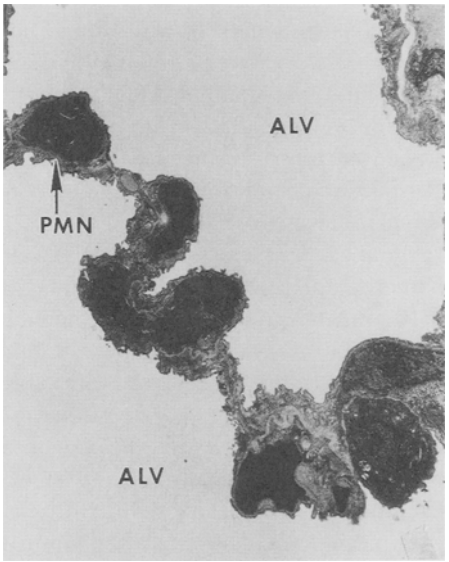


FIG. 8. Transmission electron microscopy of rat lung one hour after injection of oleic acid, stained with uranyl acetate and lead citrate. Polymorphonuclear cell (PMN, arrow) is noted in the capillary of the intraalveolar septa (magnification X: 700); ALV, alveolar space.

terial in a small vessel, possibly an OA globule (Fig. 7). At one-hour post injury, neutrophil adherence to vascular endothelium was noted (Fig. 8).

DISCUSSION

Although OA has been used extensively as a model of acute lung injury, no measurements of its distribution and concentration within the various organs during such injury have been reported. The results of measurements in this series of experiments demonstrate high concentrations of radiolabel, probably representing OA present in the lung early in lung injury.

OA in this study was toxic to endothelial cells *in vitro* at concentrations an order of magnitude less than those measured *in vivo* during experimental lung injury. This toxicity was observed as early as one minute after addition of OA to cell cultures. These findings suggest that at least one component of this form of lung injury is direct toxicity of OA to pulmonary cells. Since the endothelial cell is the first cell in the pulmonary parenchyma exposed to intravascular OA, it is likely the cell that is initially damaged.

This conclusion raises the question of the effects of OA on endothelial cells of other vascular beds. It is possible that a significant amount of OA is removed in the pulmonary vascular bed since this is the first capillary bed to be exposed after intravenous injection. This argument is supported by both the prolonged presence of radioactivity in BAL fluid in this model and the high percentage of radiolabeled free fatty acid in extracts early in lung injury. In the current experiments, the liver and kidneys contained levels of OA approximately one order of magnitude less than those found in the lung, but at levels demonstrated to be toxic *in vitro* to endothelial cells. This may not be the case in these organs *in vivo*, since a greater proportion of the oleate is found in the form of triglycerides. Hepatocytes in these *in vitro* experiments were also markedly more resistant to OA injury than both endothelial and alveolar epithelial cells. It is probable that the lung is the organ most affected by toxic effects of OA since it is the first vascular bed exposed after injection, with consequently higher tissue levels and possibly more sensitive cell types.

Ultrastructural changes in endothelial cells were identified in electron micrographs demonstrating blebbing of endothelial cells at 10 min after injury. Such endothelial blebbing in other systems has been a consistent finding of pulmonary endothelial injury (25). Similar findings have also been noted in baboon and porcine models of OA lung injury (26,27) One possible explanation for the toxicity of OA to endothelial cells is an overloading of the endothelial cell mechanisms of clearance of albumin-bound fatty acids. Both *in situ* and *in vivo*, oleic or palmitic acids bound to albumin were more rapidly taken up by endothelial cells and more rapidly transported to the albuminal surface than was albumin alone (28). It is possible that endothelial cell damage is caused by an overload of this normal mechanism for transportation of fatty acids to metabolizing cells.

Other authors have noted an injury to both pulmonary alveolar epithelial cells and endothelial cells in grossly affected areas 24 h after OA lung injury (2,29). It is obvious from the gross and microscopic appearance of the lung 10 min after injury in this study that there is disruption of the alveolar and capillary membranes in multiple hemorrhagic areas. However, electron microscopy of seemingly spared areas adjacent to the grossly involved ones demonstrates signs of selective endothelial injury with relative sparing of epithelial cells.

It is not surprising that OA exerts at least part of its effects through damage to endothelial cells, and there is evidence that this may be relevant to the etiology of several types of lung injury. Aberrations of fatty acid metabolism are seen in trauma, sepsis, pancreatitis, and fat embolism which can lead to increases in OA levels in the bloodstream. Since OA is toxic to endothelial cells at moderate concentrations, the ARDS frequently seen in such patients may be mediated in part by increased concentrations of OA.

There are several mechanisms whereby OA synthesis in the organism may be induced. During starvation or fatty acid depletion, the usual elongation and desaturation pathways producing long-chain ω6 fatty acids instead synthesize ω9 fatty acids from endogenous sources (30). In a related study on trauma patients given fat-free parenteral nutrition, OA levels in venous blood increased twofold within four weeks of injury and were significantly increased over those levels found in healthy volunteers (31).

OA metabolism is altered during experimental sepsis as well. Fuortes and co-workers (32) measured OA levels in pulmonary arterial blood of septic dogs and found that levels of OA had approximately doubled within 72 h of the onset of sepsis. These levels were comparable to those found to be toxic to endothelial cells *in vitro*; however, an exact correlation is not possible due to the difference between *in vitro* and *in vivo* conditions. The effects of tumor necrosis factor-α (TNF-α) on fatty acid metabolism and lipoprotein lipase may be responsible for these changes. TNF-α inhibits transcription of lipoprotein lipase and fatty acid synthetase in the adipocyte. The presence of TNF-α also results in an increase in hormone-sensitive lipase activity. The net effect of these changes is a reduction of stored fat and an increase in circulating triglycerides. It is plausible that such changes would result in increased OA levels during the cytokine response to endotoxemia and sepsis. Baughman and co-workers (33) measured relative concentrations of OA and other fatty acids in BAL fluid from patients with ARDS. In this study, there was a greater proportion of OA in the group of patients with ARDS as compared with the normal control group, arguing for a shift to favor production of OA.

In summary, the data presented in this study demonstrated radioactive counts consistent with levels of OA in the lung of 1×10^{-3} M 30 min after injection, mostly in the form of free fatty acid. This concentration was demonstrated to be toxic to endothelial cells *in vitro*, and damage to endothelial cells *in vivo* was further demonstrated by electron microscopy as early as 10 min after injection. These findings support the hypothesis that one of the initial events in OA lung injury is direct damage of the pulmonary vascular endothelium by OA. It is possible that OA, through endogenous production during periods of stress such as endotoxemia and trauma, may be a mediator of pulmonary injury during these events. Further studies are needed to investigate the role of OA in other forms of acute lung injury.

ACKNOWLEDGMENTS

The author wishes to thank Roger Thrall, Ph.D., for his suggestions and review of the manuscript, Nola Walker-Bupp and associates of Wesley Medical Center Electron Microscopy Laboratory, and Dou-

glas Bibus, M.S., for excellent technical assistance. Support was received from American Lung Association of Kansas #RG-065-BN Biomedical Research Support Grant Program and National Institutes of Health #BRSGS07RR06007-01. Dr. Beilman is currently a recipient of National Research Service Award #1F32HL09204-1.

REFERENCES

1. Ashbaugh, D.G., Bigelow, D.B., Petty, T.L., and Levine, B.E. (1967) Acute Respiratory Distress in Adults, *Lancet 2*, 319–323.
2. Dickey, B.F., Thrall, R.S., McCormick, J.R., and Ward, P.A. (1981) Oleic Acid Lung Induced Lung Injury in the Rat: Failure of Indomethacin Treatment or Complement Depletion to Ablate Lung Injury, *Am. J. Pathol. 103*, 376–383.
3. Derks, C.M., and Jacobovitz-Derks, D. (1977) Embolic Pneumopathy Induced by Oleic Acid, *Am. J. Pathol. 87*, 143–158.
4. Andreasson, S., Bylock, A., Smith, L., and Risberg, B. (1986) Extravascular Lung Water Measurement in Septic Sheep, *J. Surgical Res. 40*, 95–104.
5. Shiue, S.T., and Thrall, R.S. (1991) Effect of Corticosteroid Therapy on the Acute Injury and Recovery Stage of Oleic Acid Induced Lung Injury in the Rat, *Exp. Lung Res.* 17, 629–638.
6. Beilman, G.J., Clancy, J., and Olson, D.E. (1988) Natural Killer (NK) and Large Granular Lymphocyte (LGL) Response in Oleic Acid-Induced Lung Injury, *Am. Soc. Exp. Bio. Proc. 2*, A459.
7. Schuster, D.P. (1994) ARDS: Clinical Lessons from the Oleic Acid Model of Lung Injury, *Am. J. Respir. Crit. Care. Med. 149*, 245–260.
8. Binder, A.S., Kageler, W., Perel, A., Flick, M.R., and Staub, N.C. (1980) Effect of Platelet Depletion on Lung Vascular Permeability After Microemboli in Sheep, *J. Appl. Physiol. 48*, 414–420.
9. Eiermann, G.J., Dickey, B.F., and Thrall, R.S. (1983) Polymorphonuclear Participation in Acute Oleic Acid Induced Lung Injury, *Am. Rev. Respir. Dis. 128*, 845–850.
10. Baldwin, S.R., Simon, R.H., Grum, C.M., Ketai, L.H., Boxer, L.A., and Devall, L.J. (1986) Oxidant Activity in Expired Breath of Patients with Adult Respiratory Distress Syndrome, *Lancet 8471*, 11–13.
11. Bernard, G.R., Lucht, W.D., Niedermeyer, M.E., Snapper, J.R., Ogletree, M.L., and Brigham, K.L. (1984) Effect of *N*-Acetylcysteine on the Pulmonary Response to Endotoxin in the Awake Sheep and Upon *in vitro* Granulocyte Function, *J. Clin. Invest. 73*, 1722–1724.
12. Ward, P.A., Till, G.O., Hetherill, J.R., Annesley, T.M., and Kinkel, R.G. (1985) Systemic Complement Activation, Lung Injury, and Products of Lipid Peroxidation, *J. Clin. Invest. 76*, 517–527.
13. Watts, F.L., Oliver, B.L., Doran, N.H., and Thrall, R.S. (1990) Superoxide Production by Rat Neutrophils in the Oleic Acid Model of Lung Injury, *Free Radical Bio. in Med. 9*, 327–332.
14. Sugarman, H.J., Blocher, C.R., Hirsch, J.I., Strash, A.M., and Tatum, J.L. (1983) Failure of Various Agents to Decrease Oleic Acid Pulmonary Albumin Leak, *J. Surg. Res. 34*, 456–462.
15. Habliston, D.L., Whitaker, C., Hart, M.A., Ryan, U.S., and Ryan, J.W. (1979) Isolation and Culture of Endothelial Cells from the Lungs of Small Animals, *Am. Rev. Respir. Dis. 119*, 853–868.
16. D'Amore, P.A., and Klagsbrun, M. (1984) Endothelial Cell Mitogens Derived from Retina and Hypothalamus, *J. Cell Biol. 99*, 1545–1549.
17. Seglen, P.O. (1972) Preparation of Rat Liver Cells: I. Effect of Calcium on Enzymatic Dispersion in Perfused Liver, *Exp. Cell Res. 74*, 450.
18. Dobbs, L.G., Gonzalez, R., and Williams, M.C. (1986) An Improved Method for Isolating Type II Cells in High Yield and Purity, *Am. Rev. Respir. Dis. 134*, 141.
19. Denizot, F., and Lang, R. (1986) Rapid Colorimetric Assay for Cell Growth and Survival, *J. Immunol. Methods 89*, 271–277.
20. Millonig, G.J. (1961) Advantages of a Phosphate Buffer for Osmium Tetroxide Solutions in Fixation, *Appl. Physiol. 32*, 1637–1641.
21. Luft, J.H. (1961) Improvements in Epoxy Resin Embedding Methods, *J. Biophys. Biochem. 9*, 409.
22. Spurlock, B.O., Skinner, M.D., and Kattine, A.A. (1966) A Simple Rapid Method of Staining Epoxy-Embedded Specimens for Light Microscopy with the Polychromatic Stain Paragon-1301, *Am. J. Clin. Pathol. 46*, 252–258.
23. Watson, M.L. (1958) Staining of Tissue Sections for Electron Microscopy with Heavy Metals, *J. Biophys. Biochem. Cyto. 4*, 475–478.
24. Reynolds, E.S. (1963) The Use of Lead Acetate at High pH as an Electron-Opaque Stain in Electron Microscopy, *J. Cell Biol. 17*, 208–212.
25. Hurley, J.V. (1982) Types of Pulmonary Vascular Injury, *Ann. NY Acad. Sci. 384*, 269–286.
26. Johanson, W.G., Holcomb, J.R., and Coalson, J.J. (1982) Experimental Diffuse Alveolar Damage in Baboons, *Am. Rev. Respir. Dis. 126*, 142–151.
27. Grotjohan, H.P., Heijde, R.M., Wagenvoort, C.A., Wagenvoort, N., and Versprille, A. (1993) Pulmonary Vasoconstriction in Oleic Acid Induced Lung Injury. A Morphometric Study, *Int. J. Exp. Path. 74*, 347–355.
28. Galis, Z., Ghitescu, L, and Simionescu, M. (1988) Fatty Acids Binding to Albumin Increases Its Uptake and Transcytosis by the Lung Capillary Endothelium, *Eur. J. Cell Biol. 47*, 358–365.
29. Schoene, R.B., Robertson, H.T., Thorning, D.R., Springmeyer, S.C., Hlastala, M.P., and Cheney, F.W. (1984) Pathophysiologic Patterns of Resolution from Acute Oleic Acid Lung Injury in the Dog, *J. Appl. Physiol. 56*, 472–481.
30. Guarnieri, M., and Johnson, R.M. (1970) The Essential Fatty Acids, *Adv. Lipid Res. 8*, 115–168.
31. McCarthy, M., Cottam, G.L., and Turner, W.W. (1981) Essential Fatty Acid Deficiency in Critically Ill Surgical Patients, *Am. J. Surgery 142*, 747–751.
32. Fuortes, M., Polock, T.W., Holman, M.J., McMillan, M.A., Jaffe, B.M., and Scalea, T.M. (1988) Changes in Intravascular Lung Water and Fatty Acids in a Hyperdynamic Canine Model of Sepsis, *J. Trauma 28*, 1455–1459.
33. Baughman, R.P., Stein, E., MacGee, J., Rashkin, M., and Sahabjani, H. (1984) Changes in Fatty Acids in Phospholipids of the Bronchoalveolar Fluid in Bacterial Pneumonia and in Adult Respiratory Distress Syndrome, *Clin. Chem. 30*, 521–523.

[Received September 16, 1994, and in final revised form May 18, 1995; Revision accepted June 19, 1995]

Effect of Low Levels of Dietary Fish Oil on Fatty Acid Desaturation and Tissue Fatty Acids in Obese and Lean Rats

Jun-Ming Cao[a,1], Jean Paul Blond[a], Pierre Juaneda[b], Georges Durand[c] and Jean Bézard[a,*]

[a]Unité de Nutrition Cellulaire et Métabolique, Université de Bourgogne, 21004 Dijon, France, [b]Station de Recherches sur la Qualité des Aliments de l'Homme, Unité de Nutrition Lipidique, Institut National de la Recherche Agronomique, 21034 Dijon, France and [c]Laboratoire de Nutrition et Sécurité Alimentaire, Institut National de la Recherche Agronomique, 78352 Jouy en Josas, France

ABSTRACT: The effect of very low levels of dietary long-chain n-3 fatty acids on Δ6 desaturation of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), and on Δ5 desaturation of dihomo-γ-linolenic acid (20:3n-6), in liver microsomes and its influence on tissue fatty acids were examined in obese and lean Zucker rats and in Wistar rats. Animals fed for 12 wk a balanced diet containing *ca.* 200 mg of long-chain polyunsaturated n-3 fatty acids per 100 g of diet were compared to those fed the same amount of α-linolenic acid. Low amounts of long-chain n-3 fatty acids greatly inhibited Δ6 desaturation of 18:2n-6 and Δ5 desaturation of 20:3n-6, while Δ6 desaturation of 18:3n-3 was not inhibited in Zucker rats and was even stimulated in Wistar rats. Inhibition of the biosynthesis of long-chain n-6 fatty acids was reflected in a decrease in arachidonic acid (20:4n-6) content of serum lipids when fasting, and also in the phospholipid fatty acids of liver microsomes. On the contrary, heart and kidney phospholipids did not develop any decrease in 20:4n-6 during fish oil ingestion. Docosahexaenoic acid (22:6n-3), present in the dietary fish oil, was increased in serum lipids and in liver microsome, heart, and kidney phospholipids.
Lipids 30, 825–832 (1995).

The health benefits of fish oils (FO) have been extensively investigated due to observations that the incidence of coronary heart disease is low among populations that consume large amounts of fish (1). Since the active constituents of FO are likely to be the long-chain n-3 fatty acids, eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids, research has been focused on how n-3 fatty acids modify several key risk factors for coronary heart disease. These fatty acids have been shown to lower blood pressure (2), exert antithrombotic effects, protect against ventricular arrhythmias, and modify several atherogenic processes (3). In addition, EPA and DHA reduce fasting and postprandial plasma triglycerides and very low density lipoprotein (VLDL) levels in healthy and hyperlipidemic subjects (4–6), and the arachidonic acid content in cellular and lipoprotein phospholipids (PL) is decreased as it is replaced by EPA and DHA (5).

Mechanisms by which long-chain n-3 fatty acids lower plasma triglycerides have been widely studied in animal models. In humans, hypertriglyceridemia is commonly associated with obesity, so the obese hypertriglyceridemic Zucker rat constitutes a good model since these rats are overweight mainly due to hyperphagia (7), as commonly observed in humans. Hyperphagia in the obese Zucker rats evokes a high level of lipogenesis in liver (8,9), while mitochondrial fatty acid oxidation is decreased (10), giving rise to high VLDL-triglyceride secretion. Increase in Δ9 desaturation activity in liver (11,12) stimulates VLDL secretion, as it has been reported in cultured hepatocytes (13,14). When compared to its lean littermate, the obese Zucker rat presents a decreased arachidonic acid (20:4n-6) content in PL from serum, platelets, liver, heart, and kidneys (15–17), consistent with the decreased biosynthesis of arachidonic acid by Δ6 and Δ5 desaturations demonstrated in liver microsomes (16). This decrease is not compensated for by an increase in DHA (22:6n-3). However, the arachidonic acid content can be slightly augmented if the diet is rich in linoleic acid (18:2n-6) (12). When obese Zucker rats are fed a diet containing FO, hypertriglyceridemia decreases, while the activity of enzymes involved in lipogenesis decreases (18,19) and fatty acid oxidation increases (19,20).

No study has yet addressed the influence of long-chain n-3 fatty acids on the biosynthesis of arachidonic acid by Δ6 and Δ5 desaturation in the obese Zucker rat. In other phenotypes, these acids added to the diet have been reported to decrease the

[1]Visiting scholar from Mianyang Agricultural College, 621000 Mianyang, Sichuan, P.R. China.

*To whom correspondence should be addressed at Unité de Nutrition Cellulaire et Métabolique, Université de Bourgogne, BP 138, 21004, Dijon, France.

Abbreviations: 18:2n-6, Linoleic acid; 18:3n-3, α-linolenic acid; 18:3n-6, γ-linolenic acid; 20:3n-6, dihomo-γ-linolenic acid; 20:4n-6, arachidonic acid; 20:5n-3, EPA, eicosapentaenoic acid; 22:6n-3, DHA, docosahexaenoic acid; CO, control oil; FO, fish oil; PL, phospholipids; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

20:4n-6 content in tissue lipids (21,22). This question is important since in the obese Zucker rat, Δ6 and Δ5 desaturations are lower than in its lean control (16), and dietary long-chain n-3 fatty acids are likely to augment this defect. Accordingly, this study was undertaken to evaluate the influence of dietary long-chain n-3 fatty acids, in comparison with α-linolenic acid, in the obese Zucker rat. Comparisons were also made with the obese rats' lean littermates and with the commonly used Wistar rat. Additionally, tissue lipids were analyzed to see whether the presumed modifications in desaturation rates *in vivo* were reflected in their polyunsaturated fatty acid content. The low level of dietary n-3 fatty acids used in this study corresponds to what can be encountered in human diet.

MATERIALS AND METHODS

Chemicals. [1-^{14}C] Linoleic acid (50 mCi/mmol, 99% radiochemically pure), [1-^{14}C] α-linolenic acid (56 mCi/mmol, 98% pure), and [1-^{14}C] dihomo-γ-linolenic acid (47 mCi/mmol, 99% pure) were purchased from the Radiochemical Centre (Amersham, Buckinghamshire, United Kingdom). Each substrate was diluted in ethanol with the corresponding unlabeled fatty acid to a specific activity of 10 mCi/mmol. Unlabeled fatty acids, coenzymes, and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals of analytical grade were purchased from Sigma and Merck (Darmstadt, Germany).

Animals and diets. Three phenotypes of five-week-old male rats, Wistar, obese Zucker (fa/fa), and lean Zucker (Fa/-), were provided by INRA (Jouy en Josas, France). They were fed *ad libitum* for seven weeks a semisynthetic control diet (CO), containing [g/kg]: sucrose [218], maize starch [440], casein [220], cellulose [20], vitamin mixture [10.4], salt mixture [40], methionine [1.6], and a control oil mixture [50] (rapeseed oil 52%, sunflower oil 20%, and hydrogenated palm oil 30%, by weight). After this control diet, animals of each phenotype were randomly divided into two groups of n = 6 animals and fed for 12 wk on different diets. The first group of animals continued to receive the control diet (CO) and the second group was fed a fish oil-supplemented diet (FO) containing (wt%): salmon oil [15], sunflower oil [35], and hydrogenated palm oil [50]. The daily food intake was adjusted to 20 g per rat to prevent hyperphagia of obese Zucker rats. In the conditions, each rat ingested the same amount of long-chain n-3 fatty acids. As shown in Table 1, the two dietary oils contained the same amount of n-6 (linoleic acid) and n-3 fatty acids in a ratio close to 6:1. In the FO diet, 95% of the n-3 fatty acids were of longer chain (principally EPA and DHA) than α-linolenic acid. The latter was the only n-3 fatty acid of the control diet.

After an overnight fast following the dietary period, all the rats were killed by exsanguination between 0700 and 0800 hours to avoid any circadian variation in the desaturation activities (23). Blood from aorta was collected in a tube. Liver, heart, and kidneys were withdrawn, blotted on filter paper, and weighed.

TABLE 1
Fatty Acid Composition of Dietary Lipids[a]

Diet	Control oil (CO)[b]	Fish oil (FO)[c]
14:0	0.8	1.7
16:0	16.5	27.2
16:1n-7	1.2	1.5
18:0	4.4	6.0
18:1n-9	43.9	30.9
18:1n-7	3.2	1.0
18:2n-6	25.6	25.3
18:3n-3	4.3	0.2
18:4n-3		0.3
20:0	0.4	0.3
20:1n-9	0.6	1.1
20:5n-3		1.6
22:0	0.3	0.4
22:1n-11		0.5
22:5n-3		0.4
22:6n-3		1.6
SFA[d]	22.4	35.6
MUFA[e]	47.7	35.0
Total n-3	4.3	4.1

[a]The composition of fatty acids is expressed as weight percentage of total fatty acids in the oil mixture added to the three diets. For 100 g of diet, 18:2n-6 and the n-3 fatty acids represented about 1250 and 210 mg, respectively.
[b]CO: mixture [wt%] of rapeseed oil [50], sunflower oil [20], hydrogenated palm oil [30].
[c]FO: mixture [wt%] of salmon oil [15], sunflower oil [35], hydrogenated palm oil [50].
[d]SFA, saturated fatty acids.
[e]MUFA, monounsaturated fatty acids.

Desaturation assays. Liver microsomes were prepared at 4°C as previously reported (24). Briefly, about 3.5 g of liver were homogenized in a Potter-Elvehjem tube in 6 vol of 0.25 M sucrose and 0.05 M phosphate buffer (pH 7.4). The supernatant of the homogenate centrifugation at 13,000 × *g* for 20 min was recentrifuged at 105,000 × *g* for 60 min. The microsomal fraction was obtained by resuspending the second centrifugation pellet in 0.4 mL of supernatant and 0.8 mL of 0.05 M phosphate buffer (pH 7.4). The protein content of microsomes was measured according to Layne (25).

Microsomal suspensions containing 5 mg of protein were incubated in open flasks containing a high level of substrate solubilized in ethanol: 120 nmol (57 μM) of [1-^{14}C] linoleic acid, 120 nmol (57 μM) of [1-^{14}C] α-linolenic acid and 80 nmol (38 μM) of [1-^{14}C] dihomo-γ-linolenic acid. Incubations were carried out in a shaking water bath for 20 min in a total volume of 2.1 mL of incubation medium containing [mM]: phosphate buffer (pH 7.4) [72], $MgCl_2$ [4.8], CoA [0.5], ATP [3.6], and NADPH [1.2]. Incubations were stopped by adding 15 mL of chloroform–methanol (2:1, vol/vol) according to Folch *et al.* (26). After methylation of fatty acids by methanol–BF_3 (27), the conversion of the three labeled substrates into their Δ6 and Δ5 desaturation products (γ-linolenic, stearidonic, and arachidonic acid, respectively) was determined after separation by reversed-phase high-performance liquid chromatography (28). Radioactivity was measured using a

1900 TR Tri-Carb (Packard, Rungis, France). Counting efficiency was estimated by comparison with radioactive standards to be at least 95% and generally more.

Lipid analysis. Lipids from serum and liver microsomes were extracted by 15 vol of dimethoxymethane–methanol (4:1, vol/vol) (29). Those from heart and kidneys were extracted according to Folch *et al.* (26). Microsomal PL were separated by thin-layer chromatography (30). The other PL were separated by silicic acid column chromatography using Sep-Pak cartridges (31).

After addition of heptadecanoic acid (17:0), the fatty acid methyl esters were prepared by transmethylation using methanol–BF_3 (27). They were analyzed by gas–liquid chromatography. Those from serum lipids and microsomal PL were analyzed using a Becker-Packard Model 417 Gas Chromatograph equipped with a laboratory-made 30 m × 0.3 mm i.d. glass capillary column coated with Carbowax 20M (Applied Science Labs, State College, PA). Analyses were carried out at a constant temperature of 180°C with a nitrogen flow rate of 3 mL/min. The Ros injector and the flame-ionization detector were maintained at 220°C. Areas were measured by means of an ICAP 10 calculator-recorder (LTT, Paris, France). The detector response was checked with standard mixture of methyl esters (Nu-Chek-Prep, Elysian, MN), and results were expressed as mol%.

Fatty acid methyl esters from heart and kidney PL were analyzed on a Hewlett-Packard 5890 Series II Chromatograph (Palo Alto, CA), equipped with a split/splitless injector and a flame-ionization detector, both maintained at 250°C. A fused silica column (50 m × 0.25 mm i.d.) coated with DB-Wax (J&W Scientific, Folsom, CA) was used with helium as carrier gas. The oven temperature was programmed from 60 to 190°C at 20°C/min. Quantitative analysis was performed using a Chromjet integrator (Spectra Physics, Les Ulis, France) coupled to a computer equipped with a Labnet Software (Spectra Physics), and results were expressed as wt%.

Statistics. Desaturase activities and fatty acid compositions were means ± SD for n = 6 animals. After analysis of variance using Duncan's multiple-range test, means were compared in the six groups according to the least significant difference and classified according to decreasing order. Values assigned a different superscript letter were significantly different at $P < 0.05$.

RESULTS

Body weight, liver weight, and microsomal protein. As shown in Table 2, the body weight, before the nutritional experiment, of the obese Zucker rats, fed *ad libitum* was higher (+27%) than the body weights of either their lean littermates or of the Wistar rats, the latter two being equal. When fed the same amount of control diet (CO) per day (20 g), the obese Zucker and Wistar rats gained the same amount of weight (*ca.* 110 g for 12 wk of diet). The weight increase of lean Zucker rats was somewhat less. When expressed as percentage of body weight, weight gain was lower in the obese rats because of higher initial body weight. Feeding the FO diet did not modify the increase of body weight in any of the groups. The liver weight of obese rats fed the control diet was about twofold higher than in the other two groups of rats. It was also higher when expressed as percentage of body weight. In the FO diet, the obese rats had a lower liver weight, contrary to the other rats. In the control diet, the liver microsomal protein content of obese rats was slightly higher when compared to that of their lean controls. Eating the FO diet increased the protein content in the obese Zucker rats (+11%), but not in the lean rats. Liver microsomal protein content of the Wistar rats was increased (+25%) when polyunsaturated n-3 fatty acids (FO) were substituted for α-linolenic acid (CO).

Activity of desaturation in liver. Figure 1 illustrates the activity of Δ6 desaturation of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) and the activity of Δ5 desaturation

TABLE 2
Body Weight, Liver Weight, and Protein Content of Hepatic Microsomes of Rats Fed the Control Oil (CO) and the Fish Oil (FO) Diet[a]

	Zucker rats					
	Obese		Lean		Wistar rats	
	CO diet	FO diet	CO diet	FO diet	CO diet	FO diet
Body weight (g)						
Initial	439 ± 18^{d}	430 ± 17^{d}	326 ± 31^{e}	358 ± 23^{e}	336 ± 55^{e}	349 ± 35^{e}
Final	550 ± 19^{d}	543 ± 17^{d}	429 ± 45^{f}	460 ± 29^{e}	445 ± 55^{e}	467 ± 46^{e}
Body weight gain						
g	111	113	103	102	109	118
(%)[b]	25.3	26.3	31.6	28.5	34.2	34.8
Liver weight	21.3 ± 1.0^{d}	18.0 ± 0.9^{e}	10.1 ± 1.3^{f}	10.8 ± 1.2^{f}	10.9 ± 1.7^{f}	9.8 ± 1.1^{f}
Relative liver weight (%)[c]	3.9 ± 0.2^{d}	3.3 ± 0.3^{e}	2.4 ± 0.1^{f}	2.4 ± 0.1^{f}	2.5 ± 0.3^{f}	2.1 ± 0.2^{f}
Liver micosomal protein (mg/g liver)	16.8 ± 1.3^{e}	18.7 ± 1.3^{d}	15.0 ± 1.3^{f}	14.9 ± 1.0^{f}	15.9 ± 1.2^{e,f}	19.9 ± 2.6^{d}

[a]Results are means ± SD for n = 6 animals in each group. After analysis of variance, means were compared in each line and classified in decreasing order. Means assigned different superscript (d–f) letters were significantly different ($P < 0.05$).
[b]% of the initial body weight.
[c](Liver weight/body weight) × 100.

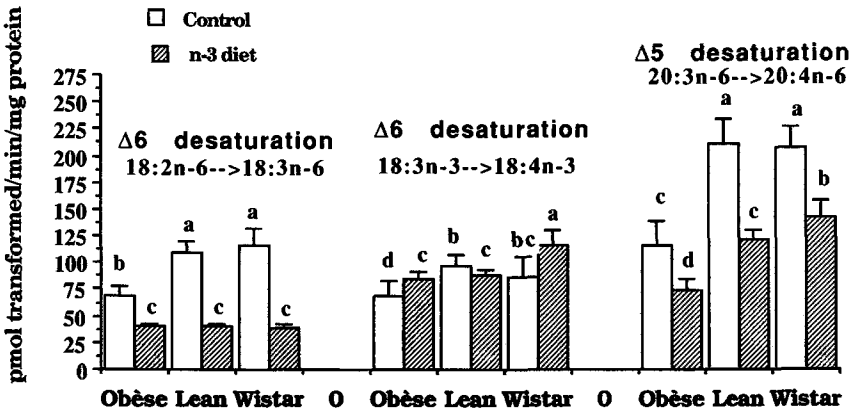


FIG. 1. Δ6- and Δ5-Desaturase activities in liver microsomes from obese, lean Zucker, and Wistar rats fed the control oil (open bars) or the fish oil (hatched bars) diet. Results were means ± SD for n = 6 animals in each group. After analysis of variance, means were compared in each desaturation. Means assigned different superscript letters were significantly different ($P < 0.05$).

of dihomo-γ-linolenic acid (20:3n-6) measured in liver microsomes of the three groups of rats fed CO or FO diets. In rats fed the control diet, the activity of 18:2n-6 Δ6 desaturation was lower in obese rats than in the other phenotypes (–40%). The activities were the same after feeding FO to the three types of rats, showing that the inhibition by FO was higher in Wistar and lean Zucker rats than in obese Zucker rats.

Δ6 Desaturation of 18:3n-3 was as active as desaturation of 18:2n-6 in obese Zucker rats fed the CO, whereas it was less active in the other two types of rats. As observed with Δ6 desaturation of 18:2n-6, the activity of Δ6 desaturation of 18:3n-3 was higher in the lean Zucker rat and in the Wistar rat than in the obese Zucker rat. The FO diet increased the 18:3n-3 desaturation activity in obese Zucker rats and to a somewhat greater degree in Wistar rats, contrary to what was observed with 18:2n-6. Δ6 Desaturation of 18:3n-3 was slightly decreased by FO feeding in lean Zucker rats.

Activity of Δ5 desaturation of 20:3n-6 was higher than the other two desaturations in the three groups of rats fed the CO. The FO diet decreased Δ5 desaturation, approximately in the same proportion in the three groups of rats, but less than the decrease in Δ6 desaturation of 18:2n-6.

If the liver weight and the liver microsomal protein content were taken into account to calculate the capacity of whole liver to desaturate (nmol of substrate converted/minute/whole liver), results showed that, with regard to the three desaturations studied, the capacity of liver to desaturate was higher in obese Zucker rats than in lean rats, contrary to what was observed when only considering the desaturase activity on a per mg protein basis. The reason is that in obese rats the liver weight and the liver microsomal protein content were higher than in lean rats.

Liver microsomal PL fatty acids. As shown in Table 3, some differences can be observed between the three types of rats fed the same control diet. When compared to its lean littermate, the obese Zucker rats had a lower percentage of n-6 fatty acids in their liver microsomal PL, 18:2n-6 being more affected than 20:4n-6; 22:6n-3 was not different. The PL fatty acid composition in Wistar rats was very close to that observed in lean Zucker rats.

The influence of FO diet was pronounced in obese Zucker rats, resulting in a decreased proportion of arachidonic acid (–20%) with a parallel increase in linoleic acid (+72%) and in dihomo-γ-linolenic acid (+75%), and an increase in DHA (+31%). As a consequence, the 20:4n-6/18:2n-6 and 20:4n-6/20:3n-6 ratios were sharply decreased by the FO diet. On the contrary, there was no effect of this diet on the monounsaturated fatty acids.

In lean Zucker rats, the same differences as in their obese littermates were observed in the proportion of n-6 and n-3 fatty acids, except that the proportion of linoleic acid was not increased, so that the 20:4n-6/18:2n-6 ratio was decreased less than in the obese rats. Results in Wistar rats were very close to those obtained in lean Zucker rats.

Serum lipid fatty acids. In obese and lean Zucker rats fed the CO diet, the differences in serum total fatty acids were very small, as can be seen in Table 4. Stearic, linoleic, and α-linolenic acid percentages were only slightly lower in obese rats. Wistar rats exhibited some differences from lean Zucker rats. Linoleic acid was higher in Wistar rats, whereas arachidonic acid and DHA were somewhat lower.

In obese Zucker rats, the FO diet did not significantly modify the arachidonic acid content, although there was a tendency to decrease. However, the 20:4n-6/18:2n-6 and 20:4n-6/20:3n-6 ratios were slightly decreased. Linolenic acid was only present in trace amounts while 22:6n-3 was higher than in the control diet group.

In lean Zucker rats, the percentage of arachidonic acid was significantly decreased by the fish oil diet so that the 20:4n-6/18:2n-6 and 20:4n-6/20:3n-6 ratios were more strikingly decreased than in the obese rats. The 22:6n-3 content was increased. The same was observed in Wistar rats, except that decrease in 20:4n-6 was not significant, similar to the findings in the obese Zucker rats.

TABLE 3
Major Fatty Acid Composition (mol%) of Liver Microsomal Phospholipids from Rats Fed the Control Oil (CO) and the Fish Oil (FO) Diet[a]

	Zucker rats				Wistar rats	
	Obese		Lean			
	CO diet	FO diet	CO diet	FO diet	CO diet	FO diet
16:0	16.1 ± 1.6[e]	18.5 ± 0.6[d]	18.4 ± 0.8[d]	19.4 ± 2.1[d]	19.1 ± 1.4[d]	18.1 ± 0.6[d]
16:1n-7	1.5 ± 0.3[d,e]	1.3 ± 0.1[e,f]	1.1 ± 0.2[f]	1.0 ± 0.2[f]	2.0 ± 0.6[d]	1.1 ± 0.1[f]
18:0	21.7 ± 2.8[d]	19.4 ± 1.5[d]	14.7 ± 2.0[e]	19.9 ± 1.0[d]	16.3 ± 2.0[e]	18.8 ± 1.6[d]
18:1n-9 + n-7	7.5 ± 0.4[d]	7.8 ± 0.4[d]	8.3 ± 0.8[d]	7.9 ± 1.0[d]	7.1 ± 2.3[d]	8.2 ± 0.6[d]
18:2n-6	3.9 ± 0.3[e]	6.7 ± 2.0[d]	7.1 ± 1.0[d]	7.3 ± 1.0[d]	8.3 ± 1.0[d]	8.1 ± 0.6[d]
20:3n-6	0.8 ± 0.1[e]	1.4 ± 0.1[d]	0.5 ± 0.1[f]	1.3 ± 0.3[d]	1.2 ± 0.2[d]	1.4 ± 0.2[d]
20:4n-6	32.4 ± 0.7[e]	25.8 ± 1.5[f,g]	35.1 ± 1.4[d]	26.8 ± 1.1[f]	32.8 ± 1.8[e]	24.7 ± 1.0[g]
22:6n-3	12.2 ± 0.2[f]	16.0 ± 1.0[d]	11.1 ± 1.4[f]	13.6 ± 1.1[e]	10.2 ± 1.4[f]	13.9 ± 2.4[d,e]
Total n-6[b]	39.1 ± 0.6[e]	35.0 ± 1.6[f]	43.5 ± 1.3[d]	38.9 ± 1.2[e]	43.4 ± 1.8[d]	35.6 ± 1.2[f]
Total n-3[c]	12.3 ± 0.2[f]	17.7 ± 1.0[d]	11.8 ± 0.3[f]	14.3 ± 1.1[e]	11.1 ± 1.4[f]	16.5 ± 2.5[d,e]
20:4n-6/18:2n-6	8.3	3.9	4.9	3.7	4.0	3.1
20:4n-6/20:3n-6	38.6	18.4	74.7	20.6	27.8	17.6
Total(n-6) + (n-3)	51.4	2.7	55.3	53.2	54.5	52.1

[a]See footnotes in Table 2. [b]Also containing 20:2, 22:4 and 22:5n-6. [c]Also containing 18:3, 20:5 and 22:5n-3.

Heart and kidney PL in Zucker rats. The PL content of heart lipids in obese and lean rats was the same whatever the diet, that is 21.8 mg/g wet weight on average. Table 5 shows that in obese rats, the FO diet did not modify the 20:4n-6 content of PL, whereas the 22:6n-3 content was increased. In lean rats, the 20:4n-6 content was neither modified while the 22:6n-3 content tended to increase on the influence of FO diet, but the difference with the CO diet was not significant. In these rats, contrary to what was observed in obese rats, the 18:2n-6 content was increased with the FO diet.

The PL content of kidney lipids in obese and lean rats was the same whatever the diet, that is 24.7 mg/g wet weight on average. Table 5 shows that in obese rats, the FO diet increased the 18:2n-6, the 20:4n-6, and the 22:6n-3 content of kidney PL. In lean rats, the 20:4n-6 content was not modified, while 18:2n-6 and 22:6n-3 were increased as in obese rats.

DISCUSSION

In this study, all the rats received the same amount of diet per day, so that the hyperphagic obese Zucker rats were food-restricted. The weight gain during the 12 wk of CO diet was the same, for obese Zucker rats and Wistar rats. Thus both groups of rats had the same food efficiency. Hyperinsulinemia that greatly stimulates lipogenesis in obese rats fed *ad libitum* (32) may not exist in food-restricted rats.

TABLE 4
Fatty Acid Composition (mol%) of Serum Lipids from Rats Fed the Control Oil (CO) and the Fish Oil (FO) Diet[a]

	Zucker rats				Wistar rats	
	Obese		Lean			
	CO diet	FO diet	CO diet	FO diet	CO diet	FO diet
16:0	20.4 ± 1.4[d]	19.7 ± 0.5[d]	19.2 ± 0.6[d]	19.2 ± 1.6[d]	18.4 ± 0.8[e]	17.0 ± 2.0[e]
16:1n-7	7.1 ± 1.3[d]	6.5 ± 0.8[d]	4.5 ± 1.3[e]	5.3 ± 0.9[e]	5.4 ± 1.9[e]	5.1 ± 0.8[e]
18:0	4.8 ± 1,4[e]	6.4 ± 0.4[d,e]	7.0 ± 1.3[d]	6.4 ± 1.1[d,e]	6.2 ± 1.9[d,e]	4.9 ± 1.7[e]
18:1n-9 + n-7	21.5 ± 3.2[d,e]	19.7 ± 0.3[e]	17.4 ± 4.3[e,f]	16.3 ± 0.9[f]	20.8 ± 2.8[d,e]	23.5 ± 1.3[d]
18:2n-6	6.5 ± 0.8[g]	7.4 ± 0.5[g]	9.4 ± 1.7[f]	11.3 ± 3.2[e,f]	12.3 ± 0.9[e]	13.6 ± 1.0[d]
18:3n-3	0.4 ± 0.1[e]	trace	0.6 ± 0.2[d]	0.2 ± 0,2[f]	0.8 ± 0.1[d]	0.2 ± 0.3[e,f]
20:3n-6	0.6 ± 0.1[d,e]	0.7 ± 0.1[d]	0.4 ± 0.2[e]	0.5 ± 0.3[d,e]	0.5 ± 0.3[d,e]	0.9 ± 0.4[d]
20:4n-6	29.0 ± 4.3[d,e]	26.7 ± 0.9[e]	30.9 ± 1.5[d]	24.6 ± 2.1[e,f]	24.7 ± 2.1[e,f]	21.7 ± 2.5[f]
22:5n-6	trace	0.4 ± 0.4[e]	trace	1.8 ± 1.5[d]	trace	1.1 ± 0.4[d]
22:5n-3	0.4 ± 0.2	trace	0.4 ± 0.2	trace	0.3 ± 0.3	trace
22:6n-3	4.5 ± 1.0[e,f]	7.1 ± 0.4[d]	5.6 ± 0.2[e]	7.2 ± 1.0[d]	4.1 ± 1.3[f]	6.5 ± 1.1[d,e]
Others	4.8	5.4	4.6	7.2	6.5	5.5
Total n-6[b]	37.7	35.6	42.0	38.5	39.5	37.7
Total n-3[c]	5.6	8.1	6.6	9.0	6.2	9.7
Total n-6/Total n-3	6.7	4.4	6.4	4.3	6.4	3.9
Total n-6 + Total n-3	43.3	43.7	48.6	47.5	45.7	47.4

[a]See footnotes in Table 2. [b]Also containing 20:2 and 22:4n-6. [c]Also containing 20:5n-3.

TABLE 5
Major Fatty Acid Composition (wt%) of Heart and Kidney Phospholipids from the Zucker Rats Fed the Control Oil (CO) and the Fish Oil (FO) Diet[a]

		Obese		Lean	
Fatty acid	Tissue	CO	FO	CO	FO
Total SFA	Heart	39.8 ± 2.6	35.8 ± 1.0	39.9 ± 2.8	36.2 ± 0.6
	Kidney	44.2 ± 1.7[d]	37.0 ± 0.3[e]	42.1 ± 1.7[d]	36.4 ± 0.4[e]
Total MUFA	Heart	14.5 ± 0.5	12.4 ± 0.3	12.5 ± 0.8	10.9 ± 0.2
	Kidney	18.4 ± 0.4[d]	15.9 ± 0.4[e]	14.7 ± 0.5	13.9 ± 0.3
18:2n-6	Heart	7.5 ± 0.3	8.2 ± 0.4	10.9 ± 0.5[e]	15.9 ± 0.4[d]
	Kidney	4.1 ± 0.1[e]	4.9 ± 0.1[d]	8.3 ± 0.2[e]	9.7 ± 0.4[d]
20:4n-6	Heart	22.2 ± 1.9	23.6 ± 04	20.6 ± 2.4	28.0 ± 0.3
	Kidney	24.2 ± 1.7[e]	30.6 ± 0.3[d]	27.3 ± 1.2	36.4 ± 0.4[d]
22:6n-3	Heart	7.6 ± 1.8[e]	13.2 ± 0.6[d]	9.3 ± 1.5	12.7 ± 0.8
	Kidney	1.8 ± 0.2[e]	3.8 ± 0.03[d]	2.0 ± 0.2[e]	3.3 ± 0.1[d]
Total n-6[b]	Heart	32.8 ± 2.4	33.7 ± 0.6	33.1 ± 2.7	36.5 ± 0.3
	Kidney	29.7 ± 1.9[e]	37.0 ± 0.3[d]	37.0 ± 0.1	39.2 ± 0.6
Total n-3[c]	Heart	9.5 ± 1.0[e]	15.0 ± 0.6[d]	10.7 ± 1.8	14.7 ± 0.9
	Kidney	2.1 ± 0.3[e]	4.6 ± 0.1[d]	2.3 ± 0.2[e]	4.8 ± 0.2[d]

[a]Results are expressed as means ± SD. They were compared in each phenotype between CO and FO diet rats and classified in decreasing order. Means assigned different superscript letters were significantly different ($P < 0.05$). Abbreviations as in Table 1.
[b]Also containing 20:2, 22:4, and 22:5n-6.
[c]Also containing 18:3, 20:5, and 22:5n-3.

When FO replaced CO in the rats' diet, the weight gain was not modified. The absence of effect in the obese rats is not in agreement with results reported by Jen *et al.* (18) in the same type of rats receiving 30 g EPA + DHA/kg diet and by Cunnane *et al.* (33) in the obese ob/ob mice. These authors observed a decreased weight gain when cod liver oil was present in the diet (100 g/kg diet). The reason may be that in our experiments, FO was added to the diet in a very low amount, about 200 mg of n-3 fatty acids per 100 g of diet. Moreover, our obese rats were food-restricted, and not fed *ad libitum* as in the cited authors' experiments.

Despite the low amount of dietary FO, the obese rats eating this diet had significantly lower liver weight, contrary to what was observed in lean Zucker and Wistar rats. This effect was probably due, in part, to a decrease in triglyceride content as observed elsewhere (20) since FO is known to strongly inhibit lipogenesis (34). In contrast, the dietary FO increased the liver microsomal protein content in obese Zucker and in Wistar rats.

With regard to liver desaturation activity, the activity of the three desaturations studied was lower in a per mg protein basis in obese Zucker rats than in lean rats fed the control diet. However, when the total liver microsomal protein was taken into account, the reverse was true, because of higher liver weight and higher amount of microsomal protein per g of liver in the obese rats. Thus, the obese rat liver possessed a higher capacity to desaturate fatty acids than livers of the lean phenotype. This confirms previous results (15), but does not explain why the lipids in several tissues contain less arachidonic acid in obese Zucker rats than in their lean controls (16). The desaturation capacity measured *in vitro* may not be totally expressed *in vivo* because of an unfavorable hormonal environment in the latter case.

The presence of FO in the diet highly decreased Δ6 and Δ5 desaturation of n-6 fatty acids in the three types of rats, in agreement with results obtained by Christiansen *et al.* (35) in Wistar rats, by Garg *et al.* (36,37) in Sprague-Dawley rats and by Grønn *et al.* (38) in isolated rat hepatocytes. We observed this effect with only about 0.75% of salmon oil in the diet. The presence of long-chain n-3 fatty acids in FO is responsible for this effect, since the total n-3 fatty acid content was the same in the control diet, but as 18:3n-3 in the latter.

Surprisingly, the presence of long-chain n-3 fatty acids in the diet did not inhibit Δ6 desaturation of 18:3n-3, despite the fact that this reaction was not needed since longer chain n-3 fatty acids were provided by the diet. This effect is contrary to that observed by Christiansen *et al.* (35) in Wistar rat also fed FO. The differential effect of dietary FO on Δ6 desaturation of 18-carbon n-3 and n-6 fatty acids would support the existence of two different desaturation enzymes or two different forms of the same enzyme.

The mechanism by which dietary long-chain n-3 fatty acids inhibit *in vitro* desaturation of n-6 fatty acids is not established in this work. One possibility may be that they depressed *in vivo* the synthesis of the desaturation enzymes in the same manner as they depressed the synthesis of the enzymes involved in lipogenesis (34). However, the presence of trace amount of free 20:5n-3 or 22:6n-3 in the isolated microsomes used for the desaturation assays may exert an allosteric product inhibition. Only experiments using specific antibodies to measure the actual amount of desaturase in microsomes and/or specific probes to measure the amount of desaturase mRNA synthesized could answer this question.

In fasting animals, serum lipids principally originate from liver, and the activity of liver Δ6 and Δ5 desaturations of n-6

fatty acids is likely to be reflected in serum lipid fatty acid composition. Indeed, the 20:4n-6 content of serum lipids in this work was generally lower after the FO diet, although not significantly in obese rats. On the contrary, there was a higher percentage of 22:6n-3, likely of dietary origin.

Since Δ6 desaturation capacity is limited in heart and kidneys (39), these organs are highly dependent on plasma lipids for n-6 and n-3 long-chain fatty acids needed for membrane PL biosynthesis. However, the analyses of heart and kidney PL fatty acids did not show any decrease in 20:4n-6 content in obese and lean Zucker rats fed the FO diet. The 20:4n-6 content was even higher in kidney PL of the obese rats. Perhaps these organs augment their uptake rate of arachidonic acid to fulfill their specific requirement in this acid, in order to maintain the physical properties of the membranes or to preserve a readily available pool of eicosanoid precursor fatty acids. On the contrary, the 22:6n-3 content was increased in PL from rats fed the FO diet. There are at least two explanations for this effect; this acid represents 39% of the dietary n-3 fatty acids, and the liver capacity to desaturate 18:3n-3 was not inhibited by FO feeding, and even increased in Wistar rats.

In contrast to heart and kidney, inhibition of Δ6 and Δ5 desaturation of n-6 fatty acids in liver microsomes was reflected in their PL fatty acid profile. In rats fed FO, the 20:4n-6 percentage was much lower than in controls. The percentage of 20:3n-6, the precursor of 20:4n-6 in the Δ5 desaturation reaction, was increased in Zucker rats, probably reflecting the sharp decrease of *in vitro* Δ5 desaturation in these animals. This effect was absent in the Wistar rats in which Δ5 desaturation inhibition was less pronounced. This shows that a good correlation between tissue fatty acid composition and desaturase activity is not always observed, as already pointed out by others (40).

Microsomal PL fatty acids closely reflected the decrease in desaturation capacity, because the desaturation products probably were rapidly incorporated into PL, as demonstrated for Δ5 desaturation *in vitro* (24). This could modify the physical and biological properties of the endoplasmic membrane and of the proteins of the desaturation systems anchored in this membrane. On the other hand, the 22:6n-3 content was increased and the total n-6 + n-3 fatty acids was not modified at all after FO feeding. This likely indicates important regulatory mechanisms to maintain an optimal membrane composition and function.

ACKNOWLEDGMENTS

The authors gratefully acknowledge O. Flety for excellent technical assistance. This work was supported by the Ministère de la Recherche et de la Technologie (Grant number 89G 0494).

REFERENCES

1. Kromhout, D. (1989) n-3 Fatty Acids and Coronary Heart Disease: Epidemiology from Eskimos to Western Populations, *J. Intern. Med. 225 (Suppl. 1)*, 47–51.
2. Knapp, H.R. (1993) Dietary Omega 3 Fatty Acids and Blood Pressure Control, in *Omega-3 Fatty Acids: Metabolism and Biological Effects* (Drevon, C.A., Baksaas, I., and Krohan, H.E., eds.) pp. 241–249, Birkhâuser Verlag, Basel/Switzerland.
3. Connor, W.E., and Connor, S.C. (1990) Diet, Atherosclerosis and Fish Oil, *Adv. Intern. Med. 35*, 139–172.
4. Harris, W.S. (1989) Fish Oils and Plasma and Lipoprotein Metabolism in Humans: A Critical Review, *J. Lipid Res. 30*, 785–807.
5. Nestel, P.J. (1990) Effects of n-3 Fatty Acids on Lipid Metabolism, *Annu. Rev. Nutr. 10*, 149–167.
6. Harris, W.S., Rothrock, D.W., Fanning, A., Inkeles, S.B., Goodnight, S.H., Illingworth, Jr., D.R., and Connor, W.E. (1990) Fish Oils in Hypertriglyceridemia: A Dose-Response Study, *Am. J. Clin. Nutr. 51*, 399–406.
7. Martin, R.J., Wandness, P.J., and Gahaga, J.H. (1978) Diurnal Changes in Serum Metabolites and Hormones in Lean and Zucker Rats, *Horm. Metab. Res. 10*, 187–192.
8. Martin, R.J. (1975) *In vivo* Lipogenesis and Enzyme Levels in Adipose and Liver Tissue from Paired-Fed Genetically Obese and Lean Rats, *Life Sci. 14*, 1447–1453.
9. Godbole, V., York, D.A., and Bloxham, D.P. (1978) Development Changes in the Fatty (fa/fa) Rat Evidence for Defective Thermogenesis Preceding the Hyperlipogenesis and Hyperinsulinemia, *Diabetologia 15*, 41–44.
10. Clouet, P., Henninger, C., Pascal, M., and Bézard, J. (1985) High Sensitivity of Carnitine Acyltransferase I to Malonyl-CoA Inhibition in Liver of Obese Zucker Rats, *FEBS Letters 182*, 331–334.
11. Wahle, K.J.W. (1975) Fatty Acid Composition and Desaturase Activity of Tissue in the Genetically Obese Zucker Rat, *Comp. Biochem. Physiol. 48*, 565–574.
12. Weekes, T.E.C., Wahle, K.J.W., and Lebaijuri, M.B. (1986) Effect of Dietary Triolein and Sunflower Oil on Insulin and Lipid Metabolism in Zucker Rats, *Lipids 21*, 220–225.
13. Wang, S.R., Infante, J., Calala, D., Petit, D., Bonnefis, M.T., and Infante, R. (1989) Lipid and Lipoprotein Synthesis in Isolated and Cultured Hepatocytes from Lean and Obese Zucker Rats, *Biochim. Biophys. Acta 1002*, 302–311.
14. Legrand, P., and Lemarchal, P. (1992) Stearyl-CoA Desaturase Activity and Triglyceride Secretion in Isolated and Cultured Hepatocytes from Genetically Lean and Fat Chickens, *Comp. Biochem. Physiol. 102B*, 371–375.
15. Escoubet, B., Griffaton, G., Guesnet, P., Lechat, P., and Lavau, M. (1987) Prostaglandin Synthesis and Membrane Fatty Acid Composition in the Heart from Obese Zucker Rats, *Biochem. Biophys. Res. Commun. 146*, 589–595.
16. Blond, J.P., Henchiri, C., and Bézard, J. (1989) Δ6 and Δ5 Desaturase Activities in Liver from Obese Zucker Rats at Different Ages, *Lipids 24*, 389–395.
17. Guesnet, Ph., Bourre, J.M., Guerre-Millo, M., Pascal, G., and Durand, G. (1990) Tissue Phospholipid Fatty Acid Composition in Genetically Lean (Fa/-) or Obese (fa/fa) Zucker Female Rats on the Same Diet, *Lipids 25*, 517–522.
18. Jen, K.L.C., Alexander M., Zhong, S., Rose, K., Lin, P.K.H., and Kasim, S.E. (1989) Liver Lowering Effect of Omega 3 Fatty Acids in Genetically Obese Zucker Rats, *Nutr. Res. 9*, 1217–1228.
19. Mohan, P.F., Phillips, F.C., and Clearly, P. (1991) Metabolic Effects of Coconut, Safflower or Menhaden Oil Feeding in Lean and Obese Zucker Rats, *Br. J. Nutr. 66*, 285–299.
20. Niot, I., Gresti, J., Boichot, J., Durand, G., Bézard, J., and Clouet, P. (1994) Effect of Dietary n-3 and n-6 Polyunsaturated Fatty Acids on Lipid-Metabolizing Enzymes in Obese Rat Liver, *Lipids 29*, 481–489.
21. De Schrijver, R., and Privett, O.S. (1982) Effects of Dietary Long Chain Fatty Acids on the Biosynthesis of Unsaturated Fatty Acids in the Rat, *J. Nutr.* 112, 619–626.

22. Nassar, B.A., Huang, Y.S., Manku, M.S., Das, U.N., Morse, N., and Horrobin, D.F. (1986) The Influence of Dietary Manipulation with n-3 and n-6 Fatty Acids on Liver and Plasma Phospholipid Fatty Acids in Rats, *Lipids 21*, 652–656.
23. De Gomez Dumm, I.N.T., De Alaniz, M.J.T., and Brenner, R.R. (1984) Daily Variations of the Biosynthesis and Composition of Fatty Acids in Rats Fed on Complete and Fat-Free Diets, *Lipids 19*, 91–95.
24. Blond, J.P., and Bézard, J. (1991) Δ5 Desaturation of Dihomo-γ-Linolenic Acid (20:3n-6) into Arachidonic Acid (20:4n-6) by Rat Liver Microsomes and Incorporation of Fatty Acids in Microsome Phospholipids, *Biochim. Biophys. Acta 1084*, 255–260.
25. Layne, E. (1957) Spectrophotometric and Turbidimetric Methods for Measuring Proteins, in *Methods in Enzymology*, pp. 447–454, Academic Press, New York.
26. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
27. Slover, H.T., and Lanza, E. (1979) Quantitative Analysis of Food Fatty Acids by Capillary Gas Chromatography, *J. Am. Oil Chem. Soc. 56*, 933–943.
28. Narce, M., Gresti, J., and Bézard, J. (1988) A Method for Evaluating the Bioconversion of Radioactive Polyunsaturated Fatty Acids by Use of Reversed-Phase Liquid Chromatography, *J. Chromatogr. 448*, 249–264.
29. Delsal, J.L. (1944) Nouveau Procédé D'extraction des Lipides du Sérum Par le Méthylal. Application Aux Microdosages du Cholestérol Total, des Phosphoaminolipides et des Protéines, *Bull. Soc. Chim. Biol. 26*, 99–105.
30. Brown, J.L., and Johnston, J.M. (1962) Radioassay of Lipid Components Separated by Thin Layer Chromatography, *J. Lipid Res. 3*, 480–481.
31. Juanéda, P., and Rocquelin, G. (1985) Rapid and Convenient Separation of Phospholipid and Non-Phosphorus Lipids from Rat Heart Using Silica Cartridges, *Lipids 28*, 40–41.
32. Malewiak, M.F., Griglio, S., Mackay, S., Lemonnier, D., and Rosselin, G. (1977) Nutritional Induced Variations in Insulinaemia, Blood Ketone Bodies and Plasma and Liver Triglycerides in Genetically Obese Rats, *Diab. Metab. 3*, 81–89.
33. Cunnane, S.C., McAdoo, K.P., and Horrobin, D.F. (1986) n-3 Essential Fatty Acids Decrease Weight Gain in Genetically Obese Mice, *Br. J. Nutr. 56*, 87–95.
34. Clark, S.D., and Jump, D.B. (1993) Regulation of Gene Expression by Dietary Fats: A Unique Role of Polyunsaturated Fatty Acids, in *Nutrition and Gene Expression* (Berdanier, C.D., and Hargrove, J.L., eds.) pp. 227–246, CRC Press, Boca Raton.
35. Christiansen, E.N., Land, J.S., Rørtveit, T., and Rustan, A.C. (1991) Effect of Dietary n-3 and n-6 Fatty Acids on Fatty Acid Desaturation in Rat Liver, *Biochim. Biophys. Acta 1082*, 57–62.
36. Garg, M.L., Sebokawa, E., Thomson, A.B.R., and Clandinin, M.T. (1988) Δ6 Desaturation Activity in Liver Microsomes of Rats Fed Diet Enriched with Cholesterol and/or ω3 Fatty Acids, *Biochem. J. 249*, 351–356.
37. Garg, M.L., Thomson, A.B.R., and Clandinin, M.T. (1988) Effect of Dietary Cholesterol and/or ω3 on Lipid Composition and Δ5 Desaturase Activity of Rat Liver Microsomes, *J. Nutr. 118*, 661–668.
38. Grønn, M., Christiansen, E., Hagve, T.A., and Christophersen, B.O. (1992) Effects of Dietary Purified Eicosapentaenoic Acid (20:5n-3) and Docosahexaenoic Acid (22:6n-3) on Fatty Acid Desaturation and Oxidation in Isolated Rat Liver Cells, *Biochim. Biophys. Acta 1125*, 35–43.
39. Brenner, R.R. (1971) The Desaturation Step in Animal Biosynthesis of Polyunsaturated Fatty Acids, *Lipids 6*, 567–571.
40. Poisson, J.P.G., and Cunnane, S.C. (1991) Long-Chain Fatty Acid Metabolism in Fasting and Diabetes: Relation Between Altered Desaturase Activity and Fatty Acid Composition, *J. Nutr. Biochem. 2*, 60–70.

[Received December 6, 1994, and in final revised form May 19, 1995; Revision accepted June 23, 1995]

Thyroid Hormone Is Required for Dietary Fish Oil to Induce Hypersecretion of Biliary Cholesterol in the Rat

William F. Prigge*, Scott R. Ketover and Roger L. Gebhard

Department of Medicine, VA Medical Center, Minneapolis, Minnesota 55417
and University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT: In the rat, both fish oil diet and thyroid hormone replacement are reported to augment bile cholesterol secretion out of proportion to bile flow or secretion of other bile lipids. We sought common mechanisms for these effects and evaluated the role of phospholipid fatty acid composition in the process. Methimazole-treated hypothyroid rats were fed low-fat chow or chow supplemented with 10% corn oil or fish oil, and were studied before and after thyroid hormone treatment. Serum, hepatic, and bile lipids were measured, phospholipid fatty acid composition determined, and hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity assayed. Fish oil diet stimulated cholesterol secretion into bile only after thyroid hormone was given, and this action was synergistic with that of thyroid hormone. Reduced serum cholesterol in fish oil-treated rats was associated with increased biliary cholesterol secretion and diminished hepatic cholesterol content. This suggests that augmented biliary cholesterol secretion may contribute to the fish oil-induced reduction of serum cholesterol. No definite relationship between hepatic or biliary phospholipid fatty acid composition and biliary secretion was apparent, although high bile cholesterol secretion was associated with a low percentage of hepatic and bile phospholipid linoleic acid.
Lipids 30, 833–838 (1995).

Dietary fish oil, with ω3 fatty acids which can be incorporated into phospholipids, potentiates biliary cholesterol secretion in the rat (1–4). Rats fed 9–10% fish oil for two to four weeks secreted more cholesterol into bile than did rats fed other lipids. Secretion increased by 50% in bile sampled acutely (2,4), by 200–300% during 24-h bile sampling (1), and by 400% in chronic bile fistula rats (3). Bile acid secretion was less affected and phospholipid secretion increased only modestly, producing saturated bile in the fish oil-fed rats (3). The facilitated cholesterol secretion was increased by bile acid infusion (3). This effect appears to be species-dependent, as dietary fish oil did not increase biliary sterol secretion in the hamster, prairie dog, or green monkey (5–7). Fish oil feeding is reported to reduce cholesterol saturation index in bile of green monkeys and humans (7,8).

Dietary fish oil also lowered blood cholesterol levels in the rats given fish oil diet (FO) (1–4). It has been suggested that augmented biliary cholesterol secretion might be responsible for the fall in lipoprotein cholesterol (1,4). Alternatively, the decreased very low density lipoprotein (VLDL) secretion which is reported during FO (9,10) might increase hepatic cholesterol available for biliary secretion in the reciprocal manner suggested by Stone and Evans (11). However, FO also lowers serum cholesterol in green monkeys and in humans without reported hypersecretion of biliary cholesterol (7,12–14).

Thyroid hormone treatment also is reported to augment biliary lipid secretion (cholesterol > phospholipid > bile acids) by hypothyroid rats (15–17). Thyroid hormone enhanced bile cholesterol secretion and hepatic cholesterol synthesis without altering hepatic cholesterol content (15,18). Fatty acid composition of rat liver microsomal and mitochondrial phospholipids, particularly arachidonic acid content, is reported to be affected by thyroid hormone status (19–21). Fatty acid desaturase and elongase enzymes convert linoleic to arachidonic acid (22). Activity of these enzymes are sensitive to hormonal and nutritional status, and differ among species (19,20,22). The current experiments evaluate whether thyroid hormone is required for dietary fish oil to produce biliary lipid hypersecretion in the rat. It also addresses whether fatty acid composition might be involved in the hypersecretion of bile cholesterol.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 125–150 g were separated into three diet groups—one fed a stock diet of Ralston Purina (St. Louis, MO) ground rodent chow with 4.5% fat by weight (low-fat diet, LF), and two groups fed chow supplemented with either 10% (w/w) corn oil (corn oil diet, CO) or FO (13.2% fat by weight). CO was obtained from Procter & Gamble (Cincinnati, OH), and FO came from Southeast Fisheries Center (Charleston, SC). LF contained 12.8% calories as fat, and CO and FO had 32% calories as fat. Because cho-

*To whom correspondence should be addressed at Research Service (151), VA Medical Center, One Veterans Drive, Minneapolis, MN 55417.

Abbreviations: ANOVA, analysis of variance; CO, corn oil diet; FO, fish oil diet; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; LF, low-fat diet; T3, triiodothyronine;VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

lesterol content of chow was 18 mg/100 g and the fish oil contained 22 mg/10 g, FO contained 36 mg cholesterol/100 g (0.1 mg/kcal). LF and CO were supplemented to 0.1 mg cholesterol/kcal (LF contained 32 mg/100 g). Animals were housed in a light-reversed room (dark 0700–1900 h; light 1900–0700 h), fed diets for three weeks, and concurrently made hypothyroid by methimazole 0.025% in the drinking water (15).

Forty-eight hours prior to study, half the animals in each group received 200 μg triiodothyronine (T3) per 100 g body weight intraperitoneally, and the remainder received vehicle only (15). The T3 dose is sufficient to saturate nuclear T3 receptors and to maintain 90% occupancy for 54 h (23). Smaller groups of animals also were studied 24 and 72 h after T3 treatment.

At time of study, animals were weighed, anesthetized with Metafane, and their common bile ducts cannulated with PE-10 tubing. At 1100 h (four hours into the dark period), bile was collected by gravity drainage into tared tubes for two hours and weighed. Additional groups of animals had bile collections fractionated into two consecutive one-hour periods. The liver was then removed, weighed, and a one-gram sample homogenized in 11 mL of 0.25M sucrose. Aliquots of whole homogenate were frozen for analysis, and the remainder used to prepare microsomes (15). Animals were killed by exsanguination and serum was saved. Total activity of 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase was assayed in microsomes as previously described (15). Protein was measured by the method of Lowry *et al.* (24). Total bile acids were measured by the procedure of Talalay (25). Bile acids were determined by chromatographic separation of methyl acetate derivatives, by the method of Duane *et al.* (26). Bile phosphatidylcholine and cholesterol were measured as previously described (16).

Total and unesterified cholesterol were extracted from tissue homogenates and serum as previously described (15). Cholesterol was quantitated by gas–liquid chromatography. Liver and microsomal total phospholipids were separated from neutral lipids for fatty acid methyl ester determination as previously described (18). Hepatic lecithin also was separated from total phospholipids by thin-layer chromatography in a chloroform/methanol/28% NH_4OH (65:25:5) system for fatty acid analysis.

Statistics. Values shown are mean ± SD for n = 3–11 animals. Data were analyzed by two-factor analysis of variance (ANOVA). When the ANOVA was significant ($P < 0.05$), means were compared by Student's *t*-test.

RESULTS

Table 1 shows relative fatty acid composition of the three diets. The predominant fatty acids in CO diet were 18:2ω6 and 18:1ω9; and 14:0, 16:0, 16:1ω7, 20:5ω3, and 22:6ω3 were increased in FO.

Table 2 shows measured parameters in each diet group, both before and 48 h after T3. At time of study, animal weights averaged 214 g and did not differ between diets. T3-treated animals had slightly smaller livers, significant for the CO and FO groups. Serum cholesterol was significantly higher for hypothyroid CO animals. Serum cholesterol fell significantly, by 22–28 mg/dl, for all diet groups after T3 treatment. Lowest post-T3 cholesterol levels occurred in FO diet animals. ANOVA showed no main effect of diet on hepatic HMG–CoA reductase activity. T3, however, did have a main effect [$F_{(1,50)} = 56.788$, $P = 0.0001$]. T3 increased activity 4–7-fold in all groups, reaching similar levels.

TABLE 1
Major Fatty Acids Expressed as mg/kcal in Stock Diet, Corn Oil-Supplemented and Fish Oil-Supplemented Diets

Fatty acid	Low-fat	Corn oil	Fish oil
14:0	0.11	0.11	1.97[a]
16:0	2.18	4.44	6.05[a]
16:1ω7	0.17	0.18	2.82[a]
18:0	0.49	0.92	1.23
18:1ω9	2.93	8.76[a]	5.52
18:2ω6	6.92	19.75[a]	6.58
18:3ω3	0.60	0.74	0.81
20:5ω3	0.15	0.14	3.59[a]
22:6ω3	0.25	0.21	2.36[a]

[a]Diet fatty acids found to be particularily enriched by addition of the fats chosen.

Bile flow and bile lipids also are shown in Table 2. No diet-specific effects on bile flow were seen. In contrast, ANOVA showed a main effect of T3 on bile flow, which increased significantly 48 h after T3 in LF and FO animals. ANOVA also showed a main effect of both T3 [$F_{(1,45)} = 43.609$, $P = 0.0001$] and diet [$F_{(2,45)} = 4.668$, $P = 0.0144$] on bile cholesterol secretion, and an interaction between T3 and diet [$F_{(2,45)} = 4.589$, $P < 0.0154$]. No diet-specific differences in bile cholesterol secretion were seen for hypothyroid animals, but bile cholesterol secretion increased markedly after T3 for all diets. Furthermore, T3-treated FO animals secreted significantly more cholesterol into bile than did the other diet groups. After T3, bile phospholipid secretion also increased significantly and similarly for all diet groups, whereas bile acid secretion increased significantly only in FO animals. No main effect of diet was seen for phospholipid or bile acid secretion.

To assure that marked bile acid depletion did not influence results during the bile collection period, additional animals in all study groups had bile collected for two consecutive one-hour periods. Bile from the second hour of drainage showed a uniform 24% reduction in bile acid secretion and 16% reductions in cholesterol and lecithin secretion, compared to the first hour. However, all bile lipid relationships in Table 2 were seen for both intervals.

To assure that the time course of T3 response was consistent for all diets, additional animals were studied at 24 and 72 h after T3. Maximal bile lipid secretion occurred 48 h after T3 in all groups, as previously observed (15,17). For all diets, bile lipid secretion 24 h after T3 was modestly increased from the hypothyroid state, and secretion 72 h after T3 was modestly reduced from 48 h, probably due to falling T3 receptor

TABLE 2
Effects of Dietary Fat and Triiodothyronine (T3) on Parameters Measured in Hypothyroid Rats[a]

Parameter	Low-fat diet (LF) (n = 9–10)	Low-fat + T3 diet (n = 7–9)	Corn oil diet (CO) (n = 8–9)	Corn oil + T3 diet (n = 8–10)	Fish oil diet (FO) (n = 7–9)	Fish oil + T3 diet (n = 10–11)
Liver weight (g)	10.1 ± 1.8	8.7 ± 1.4	9.9 ± 1.1	8.2 ± 1.2[b]	10.1 ± 1.1	8.3 ± 0.9[b]
Serum cholesterol (mM)	1.79 ± 0.54	1.22 ± 0.47[b]	2.33 ± 0.49[c]	1.71 ± 0.57[b]	1.68 ± 0.41	0.96 ± 0.34[b,d]
HMG–CoA reductase (pmol/mg protein min)	97 ± 48	331 ± 106[b]	95 ± 71	393 ± 189[b]	49 ± 16	363 ± 212[b]
Bile						
Flow (mL/kg/h)	2.16 ± 0.88	3.11 ± 0.70[b]	2.31 ± 0.60	2.71 ± 0.93	1.93 ± 0.39	3.48 ± 0.79[b]
Cholesterol (μmol/kg/h)	0.61 ± 0.22	2.40 ± 0.82[b]	0.83 ± 0.41	2.45 ± 1.11[b]	0.72 ± 0.27	4.90 ± 2.74[b,c]
Phosphatidylcholine (μmol/kg/h)	5.1 ± 2.2	11.8 ± 3.7[b]	6.2 ± 2.3	10.7 ± 3.3[b]	5.7 ± 1.6	12.8 ± 4.9[b]
Total bile acids (μmol/kg/h)	89 ± 24	108 ± 30	85 ± 24	90 ± 22	70 ± 9	98 ± 25[b]
Liver cholesterol						
Whole:total (free) (μmol/g protein)	30 ± 2.6(27)[c]	34 ± 3.2(30)[b]	47 ± 11(37)	40 ± 6.6(34)[c]	41 ± 7.4(36)	33 ± 3.1(27)[b]
Microsomal:total (free) (μmol/g protein)	51 ± 5.7(45)	54 ± 5.7(49)	49 ± 7.9(37)	60 ± 5.5(49)[b,c]	55 ± 3.2(43)	50 ± 2.5(41)[b]

[a]Values are means ± SD. HMG, 3-hydroxy-3-methylglutaryl. Significance is at least $P < 0.05$ for: [b]difference between hypothyroid vs. T3 treated; [c]different from other diet groups of comparable T3 status;[d]FO vs. CO of comparable T3 status.

occupancy. FO animals had the highest cholesterol secretion at all time points.

Individual bile acid (not shown) were not influenced by diet but showed modest changes after T3. Hypothyroid rat bile averaged 72% cholate, 15% deoxycholate, 8% muricholate, and 4% chenodeoxycholate. After T3 treatment, deoxycholate decreased to 9% ($P < 0.05$). Chenodeoxycholate rose and cholate fell after T3, but not significantly.

Finally, total and free cholesterol in whole liver homogenate and liver microsomes are shown in Table 2. Whole liver cholesterol content was greater in hypothyroid animals on the high-fat diets. After T3, hepatic cholesterol rose in LF animals, but fell significantly in FO animals. Total microsomal cholesterol of hypothyroid rats was similar on all diets, but increased significantly for CO and decreased for FO after T3. Free cholesterol represented 88–90% of whole liver and microsomal cholesterol in LF animals and 76–84% of total cholesterol in CO and FO animals, both before and after T3. Total cholesterol content, therefore, reflected both free and ester cholesterol.

Individual phospholipid species were not determined in these studies, but Table 3 shows major fatty acids in total phospholipids of whole liver homogenates and bile. The influence of FO diet is apparent, with a high percent ω3 fatty acids, and low percent 18:2ω6 and 20:4ω6. CO produced a high percent of 18:2ω6 and 20:4ω6 in total phospholipids. The ratio of saturated fatty acids to mono- and unsaturated fatty acids differed, with CO animals having the lowest proportion of saturates. This is expected, as fish oil contains a high proportion of saturated fatty acids. After T3 treatment, total phospholipid 20:4ω6 increased and 18:2ω6 decreased for all diets. This is likely a result of increased chain elongation and desaturase enzyme activities (22). T3 had no consistent effect on the ratio of phospholipid saturates to unsaturates.

Total phospholipid fatty acids in liver microsomes are not shown, but were similar to results for whole homogenate. Serum phospholipid fatty acids had less 20:4ω6 and more 16:0 than liver, but showed similar diet and T3-related changes in composition (not shown).

Fatty acid composition of hepatic phosphatidylcholine was also determined (not shown). Phosphatidylcholine had a modestly larger component of 16:0 and a smaller component of 20:4ω6. Compositional changes accompanying diet or T3 were less prominent than, but similar to, those shown for total phospholipids. Total phospholipid fatty acid compositions were chosen for display in Table 3, in order to maximally indicate the results of changes in dietary fat content and T3-induced fatty acid synthesis.

Bile phospholipids showed the expected predominance of 16:0 and 18:2ω6 fatty acids. The two fat diets caused small, but significant, changes in composition. CO increased 18:2ω6 and 20:4ω6, and FO increased ω3 fatty acids. T3 tended to increase 20:4ω6 and reduce 18:2ω6 fractions, but changes were significant only for the CO diet.

DISCUSSION

Under most conditions, bile acid secretion rate determines bile flow and the linked secretion rates of biliary cholesterol and phospholipid (27–29). Euthyroid rats fed fish oil fatty acids, alone or with ω6 fatty acids, increase bile cholesterol secretion to a greater extent than bile flow or secretion of other bile lipids (1–3). Thyroid hormone also stimulates bile cholesterol secretion in rats, out of proportion to bile flow or

TABLE 3
Effect of Diet and T3 Status on Major Phospholipid Fatty Acids, Expressed as Percent of Total[a]

Fatty acid	LF	Low-fat + T3 diet	CO	Corn oil + T3 diet	FO	Fish oil + T3 diet
Liver Homogenate	n = 10	n = 8	n = 9	n = 8	n = 6	n = 11
16:0	19.6 ± 2.2	17.6 ± 1.1[b]	15.6 ± 1.2[c]	16.1 ± 2.4[d]	19.3 ± 0.7	18.0 ± 0.9[b]
18:0	22.1 ± 2.0	21.6 ± 3.1[c]	19.4 ± 1.6	24.7 ± 1.7[b]	22.7 ± 0.4	25.9 ± 1.6[b]
18:1ω9	5.7 ± 0.9	7.8 ± 3.0[c]	7.4 ± 1.1	4.6 ± 1.1	5.5 ± 0.3	4.8 ± 0.9
18:2ω6	20.2 ± 2.6[c]	17.3 ± 1.9[b]	23.8 ± 3.2[c]	16.1 ± 0.8[b]	15.2 ± 0.5[c]	12.8 ± 0.9[b,c]
20:4ω6	17.0 ± 1.4[c]	22.3 ± 0.3[b,c]	22.0 ± 2.7[c]	27.1 ± 1.4[b,c]	12.8 ± 0.6[c]	16.6 ± 2.6[b,c]
20:5ω3	1.1 ± 0.3	0.9 ± 0.4	0.4 ± 0.2	0.2 ± 0.1	6.4 ± 0.8[c]	5.2 ± 1.7[c]
22:6ω3	7.0 ± 0.7	6.7 ± 0.8	5.3 ± 0.7	7.0 ± 0.8	10.9 ± 0.5[c]	10.9 ± 0.5[c]
Bile	n = 5	n = 5	n = 4	n = 6	n = 3	n = 7
16:0	38.1 ± 4.0	39.0 ± 1.5	31.3 ± 2.6[c]	36.9 ± 1.3[b]	35.6 ± 0.9	39.9 ± 4.1
18:0	6.0 ± 1.0	6.5 ± 1.1	5.7 ± 0.7	8.2 ± 1.1[b]	5.1 ± 0.3	7.3 ± 0.7[b]
18:1ω9	7.8 ± 1.1	9.9 ± 2.7	9.5 ± 2.5	6.1 ± 1.0[c]	7.6 ± 0.4	7.9 ± 0.9
18:2ω6	28.4 ± 3.5	26.5 ± 2.4	32.9 ± 2.2[d]	28.2 ± 1.0[b,d]	24.8 ± 1.4	23.6 ± 2.0
20:4ω6	7.4 ± 1.9	8.3 ± 1.7	10.4 ± 1.1[c]	14.2 ± 1.7[b,c]	6.9 ± 0.7	7.5 ± 2.0
20:5ω3	0.7 ± 0.3	0.4 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	4.8 ± 0.3[c]	3.4 ± 1.0[b,c]
22:6ω3	1.8 ± 0.5	1.6 ± 0.4	1.5 ± 0.1	2.2 ± 0.7	3.2 ± 0.2[c]	2.9 ± 0.9[e]

[a]Values are means ± SD. Abbreviations as in Table 2. Significance is at least $P < 0.05$ for: [b]difference between hypothyroid vs. T3 treated; [c]different from other diet groups of comparable T3 status; [d]CO vs. FO of comparable T3 status; [e]FO vs. LF of comparable T3 status.

other bile lipids (15–17). The present study tested whether T3 is required for dietary ω3 fatty acids to increase bile cholesterol secretion.

For hypothyroid rats, FO had no effect on bile lipids or bile flow when compared to LF or to a diet with equivalent amounts of CO. T3 supplementation augmented bile cholesterol secretion out of proportion to bile flow or secretion of other bile lipids in all groups, but to a much greater extent (7-fold) in FO rats than in LF or CO animals (3–4-fold). This augmentation of cholesterol secretion by FO occurred in spite of the fact that bile flow and bile acid secretion were similar for the three diets after T3. Thus, T3 was necessary for FO to stimulate bile cholesterol secretion, whereas FO had a synergistic effect on T3-induced secretion. The cholesterol hypersecretion of FO was most apparent in comparison to LF, where bile flow and secretion of phospholipids and bile acids were identical.

Dietary cholesterol is not considered to have a major influence on biliary cholesterol secretion (28,30). Because dietary cholesterol was similar for all diet groups in this study, it was not the cause of bile cholesterol hypersecretion in T3-treated FO rats. Newly synthesized cholesterol also is not felt to be a major component of biliary cholesterol secretion (28,31). In this study, hepatic HMG–CoA reductase activity was markedly stimulated by T3 treatment in all diet groups. However, synthesis cannot account for bile cholesterol hypersecretion in T3-treated FO rats, as reductase activity was identical for all diets after T3.

Both FO and T3 are known to affect serum, as well as biliary, cholesterol. Neither basal serum cholesterol level nor the quantitative reduction in blood cholesterol appeared to be sufficient to account for the twofold greater cholesterol secretion of FO rats. However, induced biliary cholesterol secretion may have produced the very low serum cholesterol levels of FO rats after T3. An alternative linkage between biliary and serum cholesterol involves decreased hepatic VLDL production during feeding of FO (9,10). Reduction in the amount of cholesterol leaving the liver *via* VLDL might then result in more hepatic cholesterol available for biliary secretion (11).

We have previously observed that T3-induced cholesterol secretion into bile did not result in reduced hepatic cholesterol content (15). This was also the case for LF and CO animals. However, the marked hypersecretion of bile cholesterol seen in T3-treated FO rats was accompanied by a significant fall in both total hepatic and microsomal cholesterol content (microsomal cholesterol increased after T3 in LF and CO rats). Thus, a primary effect of FO may have been to facilitate biliary cholesterol secretion, leading to hepatic depletion and reduced blood cholesterol. If FO caused biliary cholesterol hypersecretion solely by impairing VLDL output, hepatic cholesterol content might have been expected to increase or to remain constant.

Lecithin fatty acid species, especially 20:4ω6, have been speculated to participate in bile cholesterol distribution and cholesterol gallstone formation (6,32,33). Although dietary fatty acids altered liver and bile phospholipids modestly in this study, a relationship between fatty acid composition and bile lipid secretion was not seen. T3 increased 20:4ω6 in liver, and to a lesser extent in bile. However, FO rats had the highest biliary cholesterol secretion and the lowest 20:4ω6 levels. ω3 Fatty acid content did not correlate with cholesterol secretion either, nor did content of ω3 plus 20:4ω6. Low levels of 18:2ω6, after FO and/or T3, were associated with greater cholesterol secretion. It is possible that phospholipids containing

this fatty acid may have an inverse relationship to cholesterol secretion into bile. However, compositional changes in 18:2ω6 were small, and significant differences were seen only in liver tissue.

Bile acid species did not appear to be a factor in the FO effect. No main effect of diet on bile acids was found, either before or after T3 treatment. Hypothyroid rats did have more deoxycholate in bile than did T3-treated rats, as previously reported for humans (34). This may result from intestinal stasis and increased bacterial conversion of cholate in the hypothyroid state. We also observed an increased ratio of chenodeoxycholate/cholate after T3, consistent with human studies (34).

In summary, we confirm that rats fed FO develop biliary cholesterol hypersecretion out of proportion to bile flow or secretion of other bile lipids. The action appears to involve a thyroid hormone-dependent biliary secretion mechanism. Further studies will be required to determine whether small, diet-related changes in bile or cell membrane phospholipid fatty acid species are involved in the effect.

ACKNOWLEDGMENT

This work was supported by the VA Research Service.

REFERENCES

1. Balasubramaniam, S., Simons, S.A., Chang, S., and Hickie, J.B. (1985) Reduction in Plasma Cholesterol and Increase in Biliary Cholesterol by a Diet Rich in n-3 Fatty Acids in the Rat, *J. Lipid Res. 26*, 684–689.
2. Chautan, M., Chanussot, F., Portugal, H., Pauli, A.-M., and Lafont, H. (1990) Effects of Salmon Oil and Corn Oil on Plasma Lipid Level and Hepatobiliary Cholesterol Metabolism in Rats, *Biochim. Biophys. Acta 1046*, 40–45.
3. Smit, M.J., Verkade, H.J., Havinga, R., Vonk, R.J., Scherphof, G.L., Veld, G.I., and Kuipers, F. (1994) Dietary Fish Oil Potentiates Bile Acid-Induced Cholesterol Secretion into Bile in Rats, *J. Lipid Res. 35*, 301–310.
4. Smit, M.J., Temmerman, A.M., Wolters, H., Kuipers, F., Beijnen, A.C., and Vonk, R.J. (1991) Dietary Fish Oil-Induced Changes in Intrahepatic Cholesterol Transport and Bile Acid Synthesis in Rats, *J. Clin. Invest. 88*, 943–951.
5. Berr, F., Goetz, A., Schreiber, E., and Paumgartner, G. (1993) Effect of Dietary n-3 Versus n-6 Polyunsaturated Fatty Acids on Hepatic Excretion of Cholesterol in the Hamster, *J. Lipid Res. 34*, 1275–1284.
6. Booker, M.L., Scott, T.E., and La Morte, W.W. (1990) Effects of Dietary Fish Oil on Biliary Phospholipids and Prostaglandin Synthesis in the Cholesterol-Fed Prairie Dog, *Lipids 25*, 27–32.
7. Scobey, M.W., Johnson, F.L., Parks, J.S., and Rudel, L.L. (1991) Dietary Fish Oil Effects on Biliary Lipid Secretion and Cholesterol Gallstone Formation in the African Green Monkey, *Hepatology 14*, 679–684.
8. Berr, F., Holl, J., Jungst, D., Fischer, S., Richter, W.O., Seifferth, B., and Paumgartner, G. (1992) Dietary n-3 Polyunsaturated Fatty Acids Decrease Biliary Cholesterol Saturation in Gallstone Disease, *Hepatology 16*, 960–967.
9. Nestel, P.J., Connor, W.E., Reardon, M.F., Connor, S., Wong, S., and Boston, R. (1984) Suppression by Diets Rich in Fish Oil of Very Low Density Lipoprotein Production in Man, *J. Clin. Invest. 74*, 82–89.
10. Lang, C.A., and Davis, R.A. (1990) Fish Oil Fatty Acids Impair VLDL Assembly and/or Secretion by Cultured Rat Hepatocytes, *J. Lipid Res. 31*, 2079–2086.
11. Stone, B.G., and Evans, C.D. (1992) Evidence for a Common Biliary Cholesterol and VLDL Cholesterol Precursor Pool in Rat Liver, *J. Lipid Res. 33*, 1665–1675.
12. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) The Comparative Reductions of the Plasma Lipids and Lipoproteins by Dietary Polyunsaturated Fats: Salmon Oil Versus Vegetable Oils, *Metabolism 32*, 179–184.
13. Illingworth, D.R., Harris, S.W., and Connor, W.E. (1984) Inhibition of Low Density Lipoprotein Synthesis by Dietary Omega-3 Fatty Acids in Humans, *Arteriosclerosis 4*, 270–275.
14. Philipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S., and Illingworth, D.R. (1985) Reduction of Plasma Lipids, Lipoproteins, and Apoproteins by Dietary Fish Oils in Patients with Hypertriglyceridemia, *N. Engl. J. Med. 312*, 1210–1216.
15. Day, R., Gebhard, R.L., Schwartz, H.L., Strait, K.A., Duane, W.C., Stone, B.G., and Oppenheimer, J.H. (1989) Time Course of Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity and Messenger Ribonucleic Acid, Biliary Lipid Secretion, and Hepatic Cholesterol Content in Methimazole-Treated Hypothyroid and Hypophysectomized Rats, *Endocrinology 125*, 459–468.
16. Gebhard, R.L., Stone, B.G., Andreini, J.P., Duane, W.C., Evans, C.D., and Prigge, W.F. (1992) Thyroid Hormone Differentially Augments Biliary Sterol Secretion in the Rat. I. The Isolated-Perfused Liver Model, *J. Lipid Res. 33*, 1459–1466.
17. Gebhard, R.L., and Prigge, W.F. (1992) Thyroid Hormone Differentially Augments Biliary Sterol Secretion in the Rat. II. The Chronic Bile Fistula Model, *J. Lipid Res. 33*, 1467–1473.
18. Andreini, J.P., Prigge, W.F., Chenglie, M., and Gebhard, R.L. (1994) Vesicles and Mixed Micelles in Hypothyroid Rat Bile Before and After Thyroid Hormone Treatment: Evidence for a Vesicle Transport System for Biliary Cholesterol Secretion, *J. Lipid Res. 35*, 1405–1412.
19. Fass, F.H., and Carter, W.J. (1981) Fatty-Acid Desaturation and Microsomal Lipid Fatty-Acid Composition in Experimental Hyperthyroidism, *Biochem. J. 193*, 845–852.
20. Fass, F.H., and Carter, W.J. (1982) Fatty Acid Desaturation and Microsomal Lipid Fatty Acid Composition in Experimental Hypothyroidism, *Biochem. J. 207*, 29–35.
21. Raederstorff, O., Meier, C.A., Moser, U., and Walter, P. (1991) Hypothyroidism and Thyroxin Substitution Affect the n-3 Fatty Acid Composition of Rat Liver Mitochondria, *Lipids 26*, 781–787.
22. Brenner, R.R. (1989) Factors Influencing Fatty Acid Chain Elongation and Desaturation, in *The Role Of Fats in Human Nutrition* (Vergroesen, A.J., and Crawford, M., eds.) 2nd edn., pp. 45–79, Academic Press Limited, London.
23. Oppenheimer, J.H., Coulombe, P., and Schwartz, H.L. (1978) Nonlinear (amplified) Relationship Between Nuclear Occupancy by Triiodothyronine and the Appearance Rate of Hepatic Alphaglycerophosphate Dehydrogenase and Malic Enzyme in the Rat, *J. Clin. Invest. 61*, 987–997.
24. Lowry, O.H. Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
25. Talalay, P. (1960) Enzymatic Analysis of Steroid Hormones, *Methods Biochem. Anal. 8*, 119–143.
26. Duane, W.C., Gilberstadt, M.L., and Wiegand, D.M. (1979) Diurnal Rhythms of Bile Acid Production in the Rat, *Am. J. Physiol. 236*, R175–R179.
27. Wagner, C.I., Trotman, B.W., and Soloway, R.D. (1976) Kinetic Analysis of Biliary Lipid Excretion in Man and Dog, *J. Clin. Invest. 57*, 473–477.

28. Turley, S.D., and Dietschy, J.M. (1979) Regulation of Biliary Cholesterol Output in the Rat: Dissociation from the Rate of Hepatic Cholesterol Synthesis, the Size of the Hepatic Cholesteryl Ester Pool, and the Hepatic Uptake of Chylomicron Cholesterol, *J. Lipid Res. 20*, 923–934.
29. Lanzini, A., and Northield, T.C. (1991) Biliary Lipid Secretion in Man, *Eur. J. Clin. Invest. 21*, 259–272.
30. Duane, W.C. (1993) Effects of Lovastatin and Dietary Cholesterol on Sterol Homeostasis in Healthy Human Subjects, *J. Clin. Invest. 92*, 911–918.
31. Robins, S.J., and Brunengraber, H. (1982) Origin of Biliary Cholesterol and Lecithin in the Rat: Contribution of New Synthesis and Preformed Hepatic Stores, *J. Lipid Res. 23*, 604–608.
32. Angelico, M., Corradini, S.G., Masella, R., Alvaro, D., Cantafora, A., and Capocaccia, L. (1992) Molecular Composition of Biliary Phosphatidylcholines, as Related to Cholesterol Saturation, Transport and Nucleation in Human Gallbladder Bile, *J. Hepatology 15*, 59–66.
33. Cantafora, A., Angelico, M., DiBiase, A., Pieche, U., Bracci, F., Attili, A.F., and Capocaccia, L. (1981) Structure of Biliary Phosphatidylcholine in Cholesterol Gallstone Patients, *Lipids 16*, 589–592.
34. Angelin, B., Einarsson, K., and Leijd, B. (1983) Bile Acid Metabolism in Hypothyroid Subjects: Response to Substitution Therapy, *Eur. J. Clin. Invest. 13*, 99–106.

[Received December 16, 1994, and in final revised form July 3, 1995; Revision accepted July 24, 1995]

Cholestyramine Alters the Lipid and Energy Metabolism of Chicks Fed Dietary Medium- or Long-Chain Triacylglycerol

Remedios T. Mabayo, Mitsuhiro Furuse*, Atsushi Murai and Jun-ichi Okumura

Laboratory of Animal Nutrition, School of Agriculture, Nagoya University, Nagoya 464-01, Japan

ABSTRACT: The effects of cholestyramine, a bile acid binding polymer, on the lipid and energy metabolism of chicks given dietary medium-chain triacylglycerol (MCT) or long-chain triacylglycerol (LCT) were investigated. Chicks (from 8 to 17 days of age) were fed diets containing MCT or LCT at 200 g oil/kg diet with or without 2% cholestyramine under equalized feeding conditions. An adjusted LCT diet was formulated in order to supply another group with daily nutrients and dietary metabolizable energy (ME) equal to MCT groups, except for corn starch. ME intakes of chicks given MCT or LCT diets were reduced by cholestyramine; consequently, fat and energy retention was reduced, though the reduction was more drastic in chicks fed LCT. This was caused by a change in amounts of the fecal excretion of fat and bile acids. Cholestyramine enhanced the excretion of octanoic acid (8:0) in the feces, which suggests that bile acids are needed for 8:0 absorption. Cholestyramine affects the utilization of dietary MCT and LCT by lowering fat and energy retention in chicks. However, the effect of cholestyramine on MCT utilization was smaller than its effect on utilization of LCT.

Lipids 30, 839–845 (1995).

Medium-chain fatty acids (MCFA) can be better utilized by chicks than can long-chain fatty acids (LCFA) (1). Under *ad libitum* feeding conditions, however, medium-chain triacylglycerol (MCT) reduces food intake in chicks (2). Variations in bile acid content in the intestinal lumen may have a considerable influence on metabolic properties of the intestine. Cholestyramine (CSTY), a bile acid binding polymer, binds bile acids in the intestinal lumen and enhances their excretion, but also has other effects. In rats, CSTY increased food intake without affecting body weight change (3). It also was reported that CSTY altered the active and the passive transport properties of the intestine (4). Thomson and Keelan (4) showed that in rats, CSTY altered the jejunal uptake of fatty acid according to carbon chain length. Although Rubin *et al.* (5) have shown that MCT and long-chain triacylglycerol (LCT) were not different in their effects on bile acid secretion and excretion in rats, the effect of these triacylglycerols on bile acid secretion in chicks is not known. In chicks, the jejunum is the major site of fat absorption (6), but it was reported that MCFA were well absorbed in the absence of bile acids (7). It may be, then, that CSTY may not affect the lipid and energy metabolism in chicks fed MCT. This study, therefore, aims to investigate the effect of CSTY on the lipid and energy metabolism of chicks fed an MCT diet compared to a LCT diet under equalized (in both food amount and energy value) feeding conditions. Moreover, the effect of MCT or LCT infusion on the cystic bile flow in chicks was also studied.

*To whom correspondence should be addressed.

Abbreviations: CSTY, cholestyramine; LCFA, long-chain fatty acids; LCT, long-chain triacylglycerol; MCFA, medium-chain fatty acids; MCT, medium-chain triacylglycerol; ME, metabolizable energy; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

MATERIALS AND METHODS

Effects of CSTY on lipid metabolism of chicks fed MCT or LCT diet under controlled feeding conditions. One-day-old single-comb white leghorn male chicks (Hattori Yokei, Nagoya, Japan) were fed a commercial starter diet consisting of 21.5% crude protein and 12.1 kJ/g metabolizable energy (ME) (Marubeni Shiryo Ltd., Tokyo, Japan) for seven days. On day 8, the chicks were individually weighed after overnight food deprivation and were divided into seven groups of five chicks each. The mean body weights per group were as uniform as possible. The chicks in the seventh group were killed by cervical dislocation and were used to determine base values. The remaining six groups were individually housed in stainless-steel metabolism cages, and were fed experimental diets from day 8 to 17.

Table 1 shows the composition of experimental CSTY-free diets. The experimental diets were formulated to meet the requirement of nutrients in growing chicks (8). Oil level was set at 200 g/kg diet, corn oil was used as the LCT source, and glyceryl tricaprylate (Coconado RK®; donated by Kao Corporation, Wakayama, Japan) was used as the MCT source. Corn oil was added to the MCT diet to meet the requirement for linoleic acid (18:2n-6). Glycine was added to the diets to complement the amount of glycine contained in isolated soybean protein necessary for the growth of chicks. MCT or LCT diet was offered in which one group was fed with CSTY-free diet, and one group received 2% CSTY. Questran® (contains 44.4 % CSTY; Bristol-Myers K.K., Tokyo, Japan) was included in the diet, replacing an equal amount of corn starch. Other constituents of Questran include about 42% glucose.

Copyright © 1995 by AOCS Press

TABLE 1
Composition of Cholysteramine-Free Diets (g/kg)

Ingredient	MCT[a]	LCT[a]	Adjusted LCT diet[b]
Isolated soybean protein	226	226	244
Mineral mixture[c]	58.8	58.8	63.6
Vitamin mixture[c]	2	2	2.2
Choline chloride	1.5	1.5	1.6
L-Methionine	2.9	2.9	3.1
Inositol	1	1	1.1
Glycine	4.2	4.2	4.5
L-Threonine	1.2	1.2	1.3
Cellulose	100	100	108
Corn starch	402.4	402.4	355.6
Corn oil	20	200	215
Glyceryl tricaprylate[d]	180	0	0
Calculated ME (kJ/g)	15.9	17.2	17.3

[a]MCT, medium-chain triacylglycerol; LCT, long-chain triacylglycerol.
[b]Adjusted LCT diet provides equal daily metabolizable energy to MCT group.
[c]Mabayo *et al.* (20).
[d]Glyceryl tricaprylate (Coconado RK®; donated by Kao Corp. Wakayama, Japan).

TABLE 2
Daily Food Intake of Chicks Fed Diets Containing Medium- (MCT) or Long-Chain Triacylglycerol (LCT), or Adjusted LCT Diet With or Without 2% Cholestyramine for Ten Days

	Food intake (g)	
Days	MCT or LCT	Adjusted LCT
1	5	4.60
2	6	5.50
3	7	6.43
4	8	7.35
5	9	8.27
6	11	10.11
7	12	11.03
8	13	11.95
9	14	12.87
10	15	13.79
Accumulated consumption	100	91.90

Questran was added at 45 g/kg diet to supply 2% CSTY to MCT or LCT diet. Calculated ME values of CSTY-free diets were 15.9 and 17.2 kJ/g for MCT and LCT diets, respectively. ME of LCT was taken to be 36.5 kJ/g (9), and MCT source was 30.7 kJ/g (2). The diet was given at 100 g/bird • 10 d, which has been observed to be consumable by MCT group in a previous experiment (Mabayo, R.T., Furuse, M., and Okumura, J., unpublished data). The ME value, however, was lower in MCT diet, so an adjusted LCT diet was included in the study in which the ME was 17.3 kJ/g and was given at 91.9 g/bird • 10 d. The composition of the adjusted LCT diet was recalculated in order to provide the amount of daily nutrients equal to the groups given MCT diet. The amount of corn starch was reduced to adjust the amount of protein, vitamins, and minerals (feeding schedule is shown in Table 2). The birds in MCT diet and adjusted LCT diet had equal daily ME intake.

The chicks were killed by decapitation on day 18. The carcass was minced, frozen in liquid nitrogen, reminced and dried at 55°C for 48 h. Nitrogen in the diets and carcasses were determined by the Kjeldahl procedure, and protein was calculated as N × 6.25, where N = nitrogen. The fat of the carcasses was extracted overnight (about 16 h) with diethyl ether using a Soxhlet apparatus and measured gravimetrically. Gains in protein, fat, and energy over the experimental period were determined by subtracting values for the initial body composition from the final experimental ones. Energy content of the chick was calculated using the values 39.1 and 23.7 kJ/g for fat and protein in the body, respectively (10). Droppings were collected during the last three days to determine the N-corrected ME values of the diets (11). An automatic bomb calorimeter (Shimadzu CA-4; Shimadzu Co., Kyoto, Japan) was used to determine the gross energy content of the diets and droppings.

Total fecal bile acids were extracted by the method of Malchow-Møller *et al.* (12), and were measured enzymatically by a Total Bile Acid Test® (Wako Pure Chemical Industries, Osaka, Japan). Fecal samples were weighed and treated with 1 mL 1N HCl, and total fat was extracted by diethyl ether. Total fecal fat was measured gravimetrically by using an aliquot of the lipid extract. The remaining lipid extract was filtered, dried under a gentle stream of N gas, and the residual lipids were redissolved in 2.8% sodium methoxide to obtain the fatty acid methyl esters. The solution was incubated at 45°C for 2 h and extracted three times with 2 mL hexane. An aliquot of hexane was used for determining the fatty acid composition. The fatty acid methyl esters were analyzed on a gas chromatograph (GC-14A; Shimadzu Co.) fitted with a 25 m × 0.25 mm i.d. PEG-20M capillary column (Gasukuro Kogyo Inc., Tokyo, Japan). The chromatograph was equipped with a data system (CR-4A; Shimadzu Co.) to calculate fatty acid composition.

Effect of MCT or LCT infusion on the cystic bile flow of chicks. Single-comb white leghorn male chicks (approximately three weeks old) were selected for the measurement of diverted bile flow in this experiment. The birds were anesthetized with urethane (1.5 g/kg B.W. i.p.), and the right ventral side of the abdominal cavity was opened to expose the gall bladder and duodenum. The proximal duodenum, just below the antrum, was ligated to prevent acid entrance to the intestine. The cystic duct was cannulated with polythene tubing, and the hepatic bile duct was ligated. The cystic duct cannula was passed out of the abdominal cavity, and bile flow was measured at 10-min intervals. The basal rate of flow was established at least three hours, in which the values were discarded, prior to the administration of 1 mL MCT or LCT, which was given by slow infusion into the proximal duodenum. Results were expressed as peak percentage of basal rates, using the values at 10 min before each infusion as basal.

Statistical analysis. One-way analysis of variance was applied in Experiment 1, and Duncan's multiple range test was used to assess the significance between means. In Experiment 2, statistical significance of responses was analyzed using paired *t*-test, and the overall means between treatments was determined by *t*-test. All of the data analyses were done using a commercially available statistical package (13).

TABLE 3
Body Composition, Protein and Energy Retention, and Metabolizable Energy (ME) Value and Intake of Chicks Fed Diets Containing Medium- (MCT) or Long-Chain Triacylglycerol (LCT) With or Without Cholestyramine Under Equalized Feeding Conditions[a]

% Cholestyramine	Triacylglycerol source					
	MCT		LCT		Adjusted LCT[b]	
parameter	0	2	0	2	0	2
Body weight gain (g/d)	5.46 ± 0.20^{c,d}	5.20 ± 0.20$^{c-e,j}$	5.64 ± 0.05^{c}	4.84 ± 0.13^{e,f}	5.10 ± 0.09^{d,e,f}	4.64 ± 0.18^{f}
Body protein (g/kg body weight)	173 ± 4^{c,d}	179 ± 3^{c,j}	163 ± 3^{e}	175 ± 2^{c,d}	168 ± 3^{d,e}	170 ± 2^{d,e}
Protein retention (g/d)	1.00 ± 0.07^{c,d}	1.04 ± 0.06^{c,j}	0.907 ± 0.03^{c,d,e}	0.924 ± 0.05^{c,d,e}	0.873 ± 0.04^{d,e}	0.829 ± 0.02^{e}
Body fat (g/kg body weight)	67.6 ± 0.7^{e}	50.8 ± 3.7^{f,j}	112.0 ± 2.8^{c}	73.0 ± 2.6^{e}	95.4 ± 4.0^{d}	70.4 ± 3.0^{e}
Energy retention (kJ/d)	37.5 ± 2.0^{e}	29.4 ± 2.3^{f,j}	58.3 ± 1.9^{c}	36.7 ± 2.5^{e}	47.0 ± 1.5^{d}	32.6 ±.2.0^{e,f}
ME value (kJ/g)	15.3 ± 0.2^{d,e}	13.7 ± 0.2^{f,g,j}	16.5 ± 0.1^{c}	13.2 ± 0.3^{g}	16.1 ± 0.1^{c,d}	14.4 ± 0.7^{e,f}
ME intake (kJ/d)	153 ± 2^{d}	137 ± 2^{e,j}	165 ± 1^{c}	132 ± 3^{e}	148 ± 1^{d}	133 ± 6^{e}
Efficiency of energy utilization[h]	0.25 ± 0.01^{d,e}	0.21 ± 0.01^{e,j}	0.35 ± 0.01^{c}	0.28 ± 0.01^{d}	0.32 ± 0.01^{c}	0.25 ± 0.02^{d,e}
Heat loss[i] (kJ/d)	115 ± 1^{c}	108 ± 2^{c,d,j}	107 ± 2^{c,d}	96 ± 1^{e}	101 ± 2^{d,e}	100 ± 7^{d,e}

[a]Values are means ± SEM of five birds. Details of triacylglycerol sources are given in Table 1.
[b]Details are given in the Materials and Methods section.
[c–g]Means having different letters in a row are significantly different at $P < 0.05$.
[h]Efficiency of energy utilization = energy retention/ME intake.
[i]Heat loss = ME intake – energy retention.
[j]One missing value.

RESULTS

Effects of cholestyramine on lipid metabolism of chicks fed MCT or LCT diet under controlled feeding conditions. Table 3 shows body weight gain of chicks fed diets containing MCT or LCT with or without CSTY under equalized feeding conditions. Body weight of chicks fed the diet containing MCT were not affected by CSTY, whereas there was a negative effect in chicks fed LCT with CSTY. In the adjusted LCT diet, body weight gain was not significantly decreased by CSTY. The body weight gain in adjusted LCT diet was significantly lower than in LCT diet without CSTY.

Table 3 also shows body composition and protein and energy retention, ME value, and ME intake of chicks fed diets containing MCT or LCT with or without CSTY under equalized feeding conditions. Chicks given the LCT diet had the lowest body protein among treatments, although it was not significantly different from the adjusted LCT diet group. Body protein was not affected by the addition of CSTY in MCT or adjusted LCT diet, but was significantly increased in the LCT diet chicks. There was no significant difference in body protein retention among treatments without CSTY. Protein retention in the MCT diet was significantly higher than in the adjusted LCT diet when CSTY was given. There was no significant difference in the efficiency of protein utilization among treatments (data not shown). Body fat in chicks fell in all treatment groups with the addition of CSTY to the diets. Body fat was significantly higher in LCT diets without CSTY than in the MCT groups. Body fat was significantly lower in chicks on the adjusted LCT diet than on the LCT diet. The adjusted LCT group had significantly higher body fat and fat retention (Fig. 1) than the MCT group, in spite of equal ME intakes. In all treatments, fat retention significantly decreased with the addition of CSTY to the diet. Among the groups not given CSTY, the values were significantly higher in the adjusted LCT and LCT diets than in the MCT diet, as shown in Figure 1.

Table 3 also shows that energy retention was significantly decreased by the addition of CSTY in all treatment groups. Among diet groups without CSTY, energy retention was highest on the LCT diet. Chicks given LCT diets without CSTY had significantly higher energy retention than chicks given MCT diets. ME values significantly decreased with the addition of 2% CSTY to all diets. The ME value in the LCT diet group not receiving CSTY was significantly higher than in the MCT diet group. There was no significant difference between MCT and adjusted LCT diets without CSTY. ME intakes significantly decreased with the addition of 2% CSTY to all diets. Chicks given the LCT diet had significantly higher ME intake than those given the MCT or adjusted LCT diets without CSTY. The efficiency of energy utilization was sig-

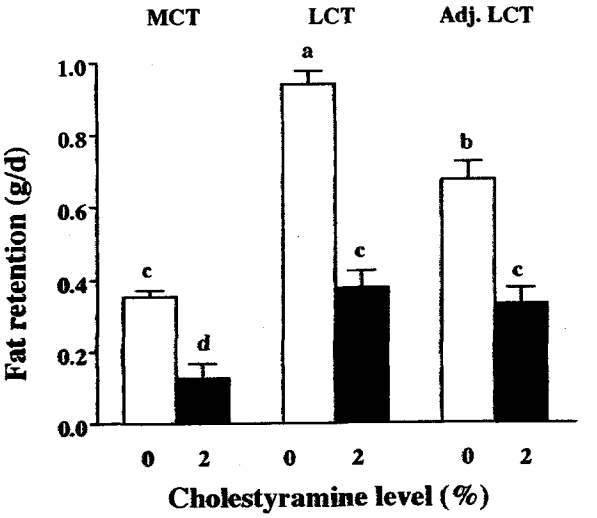


FIG. 1. Fat retention of chicks fed medium-chain triacylglycerol (MCT), long-chain triacylglycerol (LCT), or adjusted long-chain triacylglycerol (Adj. LCT) diets with or without 2% cholestyramine under equalized feeding conditions for 10 d. Values are means ± SEM of five birds. MCT with cholestyramine had one missing value. Different letters are significantly different at $P < 0.05$.

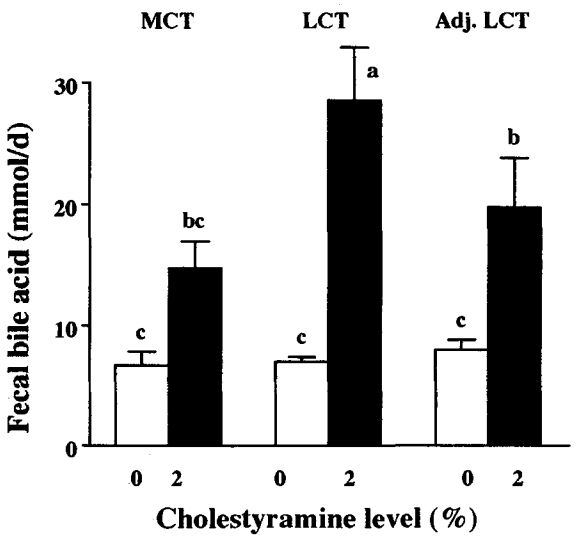


FIG. 2. Fecal bile acids of chicks fed MCT, LCT, or Adj. LCT diets with or without 2% cholestyramine under equalized feeding conditions for 10 d. Values are means ± SEM of five birds. MCT with cholestyramine had one missing value. Different superscript letters are significantly different at $P < 0.05$. Abbreviations as in Figure 1.

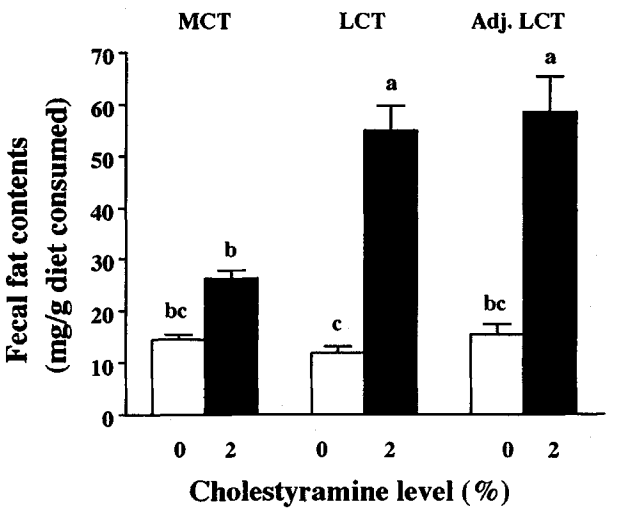


FIG. 3. Fecal fat of chicks fed MCT, LCT or Adj. LCT diets with or without 2% cholestyramine under equalized feeding conditions for 10 d. Values are means ± SEM of 5 birds. MCT with cholestyramine had one missing value. Different superscript letters are significantly different at $P < 0.05$. Abbreviations as in Figure 1.

nificantly higher in both LCT diets without CSTY than with the rest of the treatments. Heat loss was significantly higher in chicks eating the MCT diet than in those eating the adjusted LCT diet. Heat loss was reduced by CSTY in the LCT group, but was not altered by CSTY in the MCT and adjusted LCT groups.

Total fecal bile acids and fecal fat content of chicks fed diets containing MCT or LCT with or without CSTY under equalized feeding conditions are shown in Figures 2 and 3, respectively. The addition of CSTY increased fecal bile acids in all treatments, but was significantly higher in chicks fed LCT with CSTY than in those given MCT diet with CSTY. Total fecal fat content was significantly increased by the addition of CSTY in all treatment groups. In both diets containing LCT, however, the increase was about threefold, whereas with dietary MCT the increase was only about 1.5-fold (Fig. 3).

The fecal fatty acid composition of chicks fed diets con-

TABLE 4
Concentration of Fecal Fatty Acids of Chicks Fed Diets Containing Medium- (MCT) or Long-Chain Triacylglycerol (LCT) With or Without Cholestyramine Under Equalized Feeding Conditions[a]

	Triacylglycerol source cholestyramine level (%)								
	MCT			LCT			Adjusted LCT[c]		
Fatty acids[b]	0	2	(2/0)	0	2	(2/0)	0	2	(2/0)
8:0	400 ± 63^{e}	853 ± 71^{d,j}	2.1	1.5 ± 0.2^{f}	20 ± 11^{f}	13.0	6.1 ± 1.8^{f}	23 ± 6^{f}	3.7
10:0	10 ± 2^{d}	11 ± 0.6^{d,j}	1.1	1.3 ± 0.4^{f}	6.3 ± 1.8^{e}	5.0	1.2 ± 0.3^{f}	6.2 ± 1.3^{e}	5.2
12:0	1.7 ± 0.4^{e}	3.3 ± 0.2^{d,j}	1.9	0.6 ± 0.1^{e}	3.6 ± 0.9^{d}	5.8	0.6 ± 0.2^{e}	3.5 ± 0.8^{d}	5.9
14:0	2.5 ± 0.6^{e}	4.4 ± 0.3^{e,j}	1.7	1.5 ± 0.3^{e}	9.0 ± 1.9^{d}	5.9	1.7 ± 0.4^{e}	9.2 ± 1.9^{d}	5.3
16:0	30 ± 3^{e}	56 ± 3^{e,j}	1.9	61 ± 9^{e}	372 ± 68^{d}	6.1	81 ± 24^{e}	338 ± 87^{d}	4.2
16:1n-7	1.3 ± 0.2	1.6 ± 1.4^{j}	1.3	0.7 ± 0.3	3.5 ± 1.1	4.9	1.3 ± 0.2	3.5 ± 1.6	2.8
18:0	25 ± 3^{e}	44 ± 3^{e,j}	1.8	34 ± 5^{e}	221 ± 48^{d}	6.5	43 ± 12^{e}	245 ± 47^{d}	5.7
18:1n-9	51 ± 4^{e}	86 ± 3^{e,j}	1.7	162 ± 18^{e}	990 ± 161^{d}	6.1	203 ± 44^{e}	1054 ± 173^{d}	5.2
18:2n-6	79 ± 9	52 ± 38^{j}	0.6	206 ± 55	562 ± 260	2.7	242 ± 52	370 ± 165	1.5
18:3n-6	0.1 ± 0.1^{f}	1.3 ± 0.4$^{d-f,j}$	12.8	0.9 ± 0.1^{e,f}	3.3 ± 1.3^{d,e}	3.9	0.9 ± 0.3^{e,f}	3.6 ± 1.3^{d}	4.0
18:3n-3	8.8 ± 2.3^{e}	9.4 ± 0.4^{e,j}	1.1	6.7 ± 1.3^{e}	36 ± 12^{d}	5.4	7.9 ± 2.0^{e}	40 ± 7^{d}	5.1
20:3n-9	0.4 ± 0.1^{e}	4.6 ± 0.2^{e,j}	10.4	7.3 ± 0.8^{e}	49 ± 9^{d}	6.6	9.1 ± 1.9^{e}	53 ± 8.6^{d}	5.9
20:4n-6	1.5 ± 0.5^{e}	4.8 ± 1.0^{e,j}	3.2	3.5 ± 1.4^{e}	30 ± 10^{d}	8.4	5.5 ± 3.5^{e}	36 ± 15^{d}	6.6
SFA[g]	469 ± 71^{e}	971 ± 69^{d,j}	2.1	117 ± 19^{f}	631 ± 128^{e}	5.4	134 ± 38^{f}	625 ± 131^{e}	4.7
MUFA[h]	52 ± 4^{e}	87 ± 3^{e,j}	1.7	162 ± 18^{e}	993 ± 162^{d}	6.1	204 ± 44^{e}	1058 ± 174^{d}	5.2
PUFA(n-6)[i]	81 ± 9	58 ± 37^{j}	0.7	210 ± 54	595 ± 251	2.8	248 ± 49	410 ± 158	1.7

[a]Values are means ± SEM of five birds. Details in triacylglycerol sources are given in Table 1.
[b]Total of fecal fatty acids (mg) of chicks during the last three days of the experiment.
[c]Details are given in the Materials and Methods section.
[d–j]Means in a row without the same superscript letters are significantly different at $P < 0.05$.
[g]Saturated fatty acid = 14:0 + 16:0 + 18:0.
[h]Monounsaturated fatty acid = 16:1n-7 + 18:1n9.
[i]Polyunsaturated fatty acid (n-6) = 18:2n-6 + 18:3n-6 + 20:4n-6.
[j]One missing value.

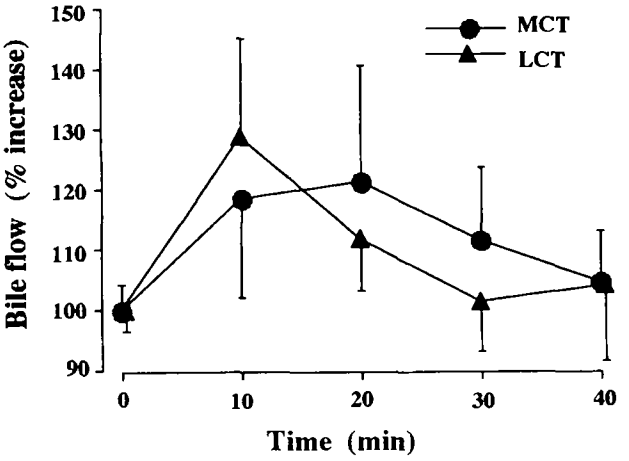


FIG. 4. Bile flow (% increase) in the cannulated cystic duct of about three-week-old single-comb white leghorn male chicks measured at 10-min intervals after duodenal infusion of MCT or LCT. Basal rate of flow was established for three hours before oil infusion in which the values were discarded. The value at 10 min before infusion was considered as basal (time 0). Values represent means ± SEM for seven chicks. No significant difference was observed between treatments. Abbreviations as in Figure 1.

taining MCT or LCT with or without CSTY under equalized feeding conditions is shown in Table 4. The values shown are total fecal of fatty acids during the last three days of the experiment. Excretion of octanoic acid (8:0) in chicks fed MCT was significantly increased by the addition of CSTY. The excretion of lauric acid (12:0) was also significantly increased in the same group. In chicks given the LCT and adjusted LCT diets, the excretion of all fatty acids listed, except for palmitoleic acid (16:1n-7) and linoleic acid (18:2n-6), was significantly increased by CSTY, including saturated and monounsaturated fatty acids. However, although the values for 18:2n-6 in the LCT with CSTY groups were increased, great variations from the means showed that significant difference was not obtained

Effect of MCT or LCT infusion on the cystic bile flow of chicks. Figure 4 shows the bile flow of chicks measured at 10-min intervals after duodenal infusion of MCT or LCT. Bile flow showed a tendency to increase at 10 min after injection of MCT or LCT. No significant difference was observed between treatments or over time.

DISCUSSION

The metabolism of dietary fat is influenced by the amount of food and energy intake, as well as by luminal bile acids. In this study, the effects of CSTY, a bile acid binding polymer, on the lipid and energy metabolism of chicks given dietary MCT or LCT, were investigated under equalized feeding conditions. LCT groups were given food amount, energy value, and CSTY level equal to MCT groups. Nutrient requirements for protein, vitamins, and minerals were adequately supplied to meet the chicks' needs. Dietary fat content, however, was very high so that the protein/energy ratio was decreased. The results presented here should be accounted for by the amount and type of dietary fat included in the diet.

CSTY is an anion exchange polymer which irreversibly binds intraluminal bile acids, as well as other components of normal luminal micelles, including phospholipid, monoacylglycerol, fatty acid, and cholesterol (14). In the present study, the dietary levels of CSTY and triacylglycerol used were 2 and 20%, respectively. Body weight gain was reduced by CSTY in chicks fed LCT, but was not altered in those fed diets with MCT. At the same level of dietary triacylglycerol, CSTY evidently affected the digestive process of chicks given LCT diet, but not MCT diet. Even at the same ME intake, lower body weight gain was attained by chicks given the adjusted LCT diet compared to those given the MCT diet when CSTY was added. Different factors discussed below may explain this finding.

Under free-feeding conditions, an increasing level of dietary MCT led to decreased fat retention as food intake was decreased (2). In the equalized feeding experiment, MCT also decreased fat retention compared to LCT. MCFA are metabolized faster in the liver and are oxidized completely (15), whereas LCFA are deposited in the tissues, resulting in increased energy retention. CSTY decreased body fat and fat retention of chicks either fed the MCT or LCT diets; consequently, energy retention was decreased. However, the drastic decrease of fat and energy retention in chicks fed the LCT diet with CSTY were great factors to consider in the reduced body weight gain. CSTY binds bile acids, decreases fatty acid absorption, and thus decreases the availability of dietary fatty acids for deposition in the body. MCFA were well absorbed by chicks in the absence of bile acids (7). It also was shown by Clark and Holt (16) that maximal steady-state absorption of MCT by rats was the same after total bile diversion as it was before. However, fat retention in chicks given the MCT diet decreased as CSTY was added. Micelle formation may not be necessary for the solubilization of MCFA if the dietary concentration is low, but bile acids may be important when the MCT concentration in the diet is high, as shown by the increased fecal fat and fecal octanoic acid (8:0) concentration (Table 4) in the MCT diet with added CSTY.

Fecal fat was increased by the addition of CSTY in the MCT diet, but it was not as drastic as in chicks given the LCT diet. The calculated ME value of the LCT diet was higher than that of the MCT diet. Furuse *et al.* (2) have shown that ME of the MCT source used in this study was lower than that of LCT (9). The addition of CSTY decreased ME values of all the diets due to incomplete solubilization of dietary fat. Thus, ME intakes also were decreased. The chicks given the MCT diet had higher heat loss than those given the adjusted LCT diet. Intravenous infusion of MCT emulsion increased energy expenditure in rats (17) and in humans (18). This factor also may be responsible for the higher heat loss in chicks fed the MCT diet compared to those fed the adjusted LCT diet, despite the same energy intake (Table 3). The chicks given the LCT diet with CSTY had a lower heat loss with reduced ME intake, which may sug-

gest that they saved more of the available energy from the LCT diet for storage.

In another experiment, bile flow was measured after cystic duct cannulation in chicks to evaluate the effect of MCT or LCT on the rate of bile flow. Diverted bile flow from the cystic duct was not significantly different between MCT or LCT administration (Fig. 4). Bile acid secretion in this experiment was not different, just as Rubin *et al.* (5) have demonstrated that lipid infusion of MCT or LCT did not affect bile flow and bile acid secretion in rats. Bile acid excreted in chicks given MCT diet with CSTY was lower than in those given diets containing LCT, and the reason for this is not understood.

The concentration of fecal fatty acids in the fecal fat (Table 4) may clarify the effect of bile acid binding by CSTY, according to the length of fatty acid carbon chain. It is shown in this study that CSTY did not enhance bile acid excretion as much during MCT feeding as during LCT feeding, but the reduced excretion of bile acids in the feces enhanced the excretion of 8:0 from chicks given MCT diet. This result raises questions in regard to the absorption mechanism of this fatty acid, as the excretion increase implies that bile acids may be needed for its absorption in large amounts. There were reports that 8:0 can be absorbed in the absence of bile acids (7,19); however, we have shown that 8:0 was excreted in the feces in a dose-dependent manner as dietary 8:0 increased (20). Decanoic acid (10:0), myristic acid (14:0), and palmitic acid (16:0) excreted by chicks given the MCT diet could be from endogenous sources. The excretion of these fatty acids increased when CSTY was added into either of the diets containing LCT, which could be due to their presence in the diet and lack of complete emulsification in the lumen. Corn oil, which was used as the LCT source, contained about 27% oleic acid (18:1n-9) and about 58% linoleic acid (18:2n-6). The concentrations of 18:1n-9 and 18:2n-6 in the fecal fat may suggest differences in absorption between these two fatty acids. The addition of CSTY did not markedly alter the absorption of 18:2n-6, which may indicate either that the micellar phase is insignificant for the absorption of this fatty acid when the bile acid concentration is low, or that CSTY is selective in binding fatty acids in the micellar phase. Linoleic acid has distinctive transport mechanisms, such as intestinal absorption through simple passive diffusion, as has been demonstrated in different species (21–23). Other LCFA, including 18:1n-9, were increasingly excreted when bound bile acids were excreted as fewer micelles were formed and fatty acids were less solubilized into micelles, which resulted in reduced fatty acid absorption.

In summary, it was concluded that CSTY alters the lipid and energy metabolism of chicks fed either MCT or LCT, as fat and energy retention of chicks was lowered, perhaps by prevention of micelle formation and bile acid reabsorption. However, the effect of CSTY on MCT utilization was less striking than its effect on that of LCT.

REFERENCES

1. Renner, R., and Hill, F.W. (1961) Utilization of Fatty Acids by the Chicken, *J. Nutr. 74*, 259–264.
2. Furuse, M., Mabayo, R.T., Kita, K., and Okumura, J. (1992) Effects of Dietary Medium Chain Triglycerides on Protein and Energy Utilisation of Chicks, *Br. Poult. Sci. 33*, 39–47.
3. Keelan, M., and Thomson, A.B.R. (1991) Feeding Diets Containing 2% Cheno- or Urso-Deoxycholic Acid or Cholestyramine to Rats for Two Weeks Alters Intestinal Morphology and Bile Acid Absorption, *Can. J. Physiol. Pharmacol. 69*, 592–598.
4. Thomson, A.B.R., and Keelan, M. (1987) Feeding Rats Diets Containing Cheno- or Ursodeoxycholic Acid or Cholestyramine Modifies Intestinal Uptake of Glucose and Lipids, *Digestion 38*, 160–170.
5. Rubin, M., Halpern, Z., Livoff, A., Weenberg, A., Tietz, A., Antebi, E., and Lichtenberg, D. (1992) The Effect of Short-Term Lipid Infusion on Liver Function and Biliary Secretion in Rats, *Lipids 27*, 321–325.
6. Hurwitz, S., Bar, A., Katz, M., Sklan, D., and Buowski, P. (1973) Absorption and Secretion of Fatty Acids and Bile Acids in the Intestine of the Laying Fowl, *J. Nutr. 103*, 543–547.
7. Garret, R.L., and Young, J.R. (1975) Effect of Micelle Formation on the Absorption of Neutral Fat and Fatty Acids by the Chicken, *J. Nutr.105*, 827–838.
8. Mabayo, R.T, Furuse, M., Yang, S.-I., and Okumura, J. (1992) Medium Chain Triacylglycerols Enhance Release of Cholecystokinin in Chicks, *J. Nutr. 122*:1702–1705.
9. Scott, M.L., Nesheim, M.C., and Young, R.J. (1982) in *Nutrition of the Chicken*, 3rd edn., pp. 482–494, M.L. Scott and Associates, New York.
10. Fraps, G.S. (1946) Composition and Productive Energy of Poultry Feeds and Rations, *Texas Agric. Exp. Sta. Bull. 678*, 5–38.
11. Hill, F.W., and Anderson, D.L. (1958) Comparison of Metabolizable Energy and Productive Energy Determinations with Growing Chicks, *J. Nutr. 64*, 587–603.
12. Malchow-Møller, A., Arffman, S., Larusso, N.F. and Krag, E. (1982) Enzymatic Determination of Total 3α-Hydroxy Bile Acids in the Faeces, *Scand. J. Gastroenterol. 17*, 331–333.
13. SAS (1985) *SAS User's Guide: Statistics*, SAS Institute, Inc., Cary.
14. Vahouny, G.V., Tombes, R., Cassidy, M.M., Kritchevsky, D., and Gallo, L. (1980) Dietary Fibers: V. Binding of Bile Salts, Phospholipids and Cholesterol from Mixed Micelles by Bile Acid Sequestrants, *Lipids 15*, 1018–1021.
15. Bach, A.C., and Babayan, V.K. (1982) Medium-Chain Triglycerides: An Update, *Am. J. Clin. Nutr. 36*, 950–962.
16. Clark, S.B., and Holt, P.R. (1968) Rate-Limiting Steps in Steady-State Intestinal Absorption of Trioctanoin-1-^{14}C; Effect of Biliary and Pancreatic Flow Diversion, *J. Clin. Invest. 47*, 612–622.
17. Lasekan, J.B., Rivera, J., Hirvonen, M.D., Keesey, R.E., and Ney, D.M. (1992) Energy Expenditure in Rats Maintained with Intravenous or Intragastric Infusion of Total Parenteral Nutrition Solutions Containing Medium- or Long-Chain Triglyceride Emulsions, *J. Nutr. 122*, 1483–1492.
18. Hill, J.D., Peters, J.C., Yang, D., Sharp, T., Kaler, M., Abumrad, N.N., and Greene, H.L. (1989) Thermogenesis in Humans During Overfeeding with Medium-Chain Triglycerides, *Metabolism 38*, 641–648.
19. Greenberger, N.J., Rodgers, J.B., and Isselbacher, K.J. (1966) Absorption of Medium and Long Chain Triglycerides: Factors Influencing Their Hydrolysis and Transport, *J. Clin. Invest. 45*, 217–227.

20. Mabayo, R.T., Furuse, M., Murai, A., and Okumura, J. (1994) Interactions Between Medium Chain and Long Chain Triacylglycerols in the Lipid and Energy Metabolism in Growing Chicks, *Lipids 39*, 139–144.
21. Chow, S.L., and Hollander, D. (1979) A Dual, Concentration-Dependent Absorption Mechanism of Linoleic Acid by Rat Jejunum *in vitro*, *J. Lipid Res. 20*, 349–356.
22. Stremmel, W. (1988) Uptake of Fatty Acids by Jejunal Mucosal Cells Is Mediated by a Fatty Acid Binding Membrane Protein, *J. Clin. Invest. 82*, 2001–2010.
23. Ling, K.Y., Lee, H.Y., and Hollander, D. (1989) Mechanisms of Linoleic Acid Uptake by Rabbit Small Intestinal Brush Border Membrane Vesicles, *Lipids 24*, 51–55.

[Received March 3, 1995, and in final revised form May 29, 1995; Revision accepted June 27, 1995]

Resistant Starch Is More Effective Than Cholestyramine as a Lipid-Lowering Agent in the Rat

Hassan Younes*, Marie-Anne Levrat, Christian Demigné and Christian Rémésy

Laboratoire des Maladies Métaboliques, INRA de Clermont-Ferrand/Theix, 63122 St-Genès-Champanelle, France

ABSTRACT: Amylase-resistant starch (RS) represents a substrate for the bacterial flora of the colon, and the question arises as whether RS shares with soluble fibers common mechanisms for their lipid-lowering effects. It is uncertain whether a cholesterol-lowering effect depends basically on an enhanced rate of steroid excretion or whether colonic fermentations also play a role in this effect. In the present study, the effect of RS (25% raw potato starch), of a steroid sequestrant (0.8% cholestyramine), or both were compared on bile acid excretion and lipid metabolism in rats fed semipurified diets. RS diets led to a marked rise in cecal size and the cecal pool of short-chain fatty acids (SCFA), as well as SCFA absorption; cholestyramine did not noticeably affect cecal fermentation. Whereas cholestyramine was particularly effective at enhancing bile acid excretion, RS was more effective in lowering plasma cholesterol (–32%) and triglycerides (–29%). The activity of 3-hydroxy-3-methylglutaryl–CoA reductase was increased fivefold by cholestyramine and twofold by RS. This induction in rats fed RS diets was concomittant to a depressed fatty acid synthase activity. In rats fed the RS diet, there was a lower concentration of cholesterol in all lipoprotein fractions, especially the (d = 1.040 – 1.080) fraction high-density lipoprotein (HDL_1), while those fed cholestyramine had only a significant reduction of HDL_1 cholesterol. In contrast to cholestyramine, RS also depressed the concentration of triglycerides in the triglyceride-rich lipoprotein fraction. There was no noticeable synergy between the effects of RS and cholestyramine when both were present in the diet. This suggests that the cholesterol-lowering effect of RS is not limited to its capacity to enhance bile acids excretion. The difference between RS and cholestyramine could relate to the capacity of fermentation end-products to counteract the upregulation of cholesterol and bile acid biosynthesis. Thus, in the absence of fermentation in the large intestine, a high rate of bile acids excretion is not always sufficient to elicit a cholesterol-lowering effect.

Lipids 30, 847–853 (1995).

The possibility of lowering plasma cholesterol by interfering with the absorption of cholesterol or bile acids has been extensively investigated using either compounds of natural origin [e.g., gel-forming fibers, resistant starch (RS), phytosterols, and saponins] or synthetic sequesterants such as cholestyramine (for review, see Ref. 1). Starch is generally the major constituent of the human diet and has long been regarded as almost entirely digested in the upper part of the digestive tract. In fact, some starches contain a significant proportion of amylase-RS and, in humans, RS may represent a susbtantial part of the polysaccharides entering the colon (2–4). These substrates are broken down by the colonic microflora, essentially to short-chain fatty acid (SCFA), which allows the recovery of a part of the chemical energy of RS by the host.

A cholesterol-lowering effect of potato starch (which is partly resistant in its native form) has been reported in rodents (5–8). Furthermore, it has been shown that amylomaize starch is very efficient at depressing plasma cholesterol in obese or in lean rats (9). In humans, a high amylose starch intake has been found to lower plasma cholesterol and triglycerides concentrations, compared to a diet rich in amylopectin starch (10); furthermore, it has been reported that a high amount of cornstarch in the diet also may reduce fat accretion (11).

The cholesterol-lowering effect of fermentable fiber may be explained by various mechanisms: (i) inhibition of diffusion of cholesterol and bile acids at the microvillous boundary layer, (ii) steroid-binding capacities in the small intestine, (iii) impairment of the passive reabsorption in the large intestine by insolubilization of bile acids (by acidification of luminal pH, microbial dehydroxylation to less polar metabolites, or entrapping on various insoluble structures), and (iv) metabolic effects, especially on hepatic lipid metabolism.

The question arises as to whether RS share with soluble fibers some common mechanisms for their lipid-lowering effects. There is little doubt that RS has a low gel-forming capacity, compared with certain soluble fibers (12), but it has been shown recently that RS has a capacity to form inclusion complexes with bile acids (due to a helix structure) (13). It is still debated as to whether a cholesterol-lowering effect depends basically on an enhanced rate of steroid excretion, and whether colonic fermentations play a role in this effect. The present study, therefore, compared the effect of RS (raw potato starch) with that of cholestyramine (a steroid sequesterant) on bile acid excretion and lipid metabolism in rats fed semipurified diets. Using diets containing both compounds, it also was examined whether these two agents had complementary antagonistic effects on plasma cholesterol.

*To whom correspondence should be addressed.

Abbreviations: ANOVA, analysis of variance; FAS, fatty acid synthase; HDL, high-density lipoprotein; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; RS, resistant starch; SCFA, short-chain fatty acid; TGRLP, triglyceride-rich lipoprotein.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Animals and diets. Forty male Wistar rats (IFFA-CREDO, l'Arbresle, France) weighing about 160 g were used. The animals were housed two per cage (wire-bottomed to limit coprophagy) and fed a semipurified moistened powder diet that contained the following (by dry weight): 15% casein (L. François, Saint-Maur, France), 5% peanut oil (CIO, Genay, France), 6% mineral mixture (UAR, Villemoisson/Orge, France), and 1% vitamin mixture (UAR). In the control diet, the carbohydrate supply was wheat starch (73%). In the experimental groups, a part of wheat starch was replaced by 25% raw potato starch (L. François) or by 0.8% cholestyramine (Sigma, St. Louis, MO) or by mixture of both. The rats were maintained in temperature-controlled rooms (22°C) with the dark period from 2000 to 0800 h. The daily food consumption and body weight were recorded every three days during the first week, then daily during the last week. Feces were collected and weighed over four consecutive 24-h periods for studies on bile acids and neutral sterol excretion. Animals were maintained and handled according to the recommendations of the Institutional Ethic Commitee (Clermont-Ferrand University, St. Gene's-Champanelle, France).

Sampling procedures. Rats were sampled just after the dark period, at a time when cecal fermentations are still very active (between 0800 and 0900 h). After anesthesia (40 mg/kg of sodium pentobarbital), rats were maintained on a warming plate at 37°C. An abdominal incision was made, and blood was withdrawn from the portal vein and then from the abdominal aorta. The blood was placed in microfuge tubes containing heparin and centrifuged at 10,000 × *g* for 2 min. Plasma samples were kept at 4°C for lipid and lipoprotein analysis. A portion of liver was freezed-clamped and stored at −80°C for the measurement of fatty acid synthase (FAS) activity. For microsome preparation, two grams of liver were quickly homogenized in 4 mL of an ice-cold buffer 1 (50 mmol/L Tris-hydrochloride, 250 mmol/L sucrose, 50 mmol/L EDTA, 2 mmol/L dithiothreitol, and 2 µmol/L leupeptin, pH 7.2) with a Potter-Elvejhem homogeneizer (Braun, Melsungen, Germany) at moderate speed. The homogenate was first centrifuged at 10,000 × *g* for 15 min at 4°C; the resulting supernatant was then centrifuged at 100,000 × *g* for 60 min at 4°C. Pellets were resuspended in 2 mL of chilled buffer 1. The centrifugation procedure was repeated, and the resulting pellets homogenized in 1 mL of buffer 2 (100 mmol/L sucrose, 50 mmol/L KCL, 40 mmol/L K phosphate, 30 mmol/L EDTA, 1 mmol/L dithiothreitol, pH 7.2). The microsomal preparation was stored at −80°C until measurement of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase activity. The protein content of the microsomes was determined using the Pierce BCA reagent kit (Interchim, Montluçon, France).

After blood sampling, the cecum with its contents was removed and weighed. Duplicate samples were collected into 2 mL microfuge tubes that were immediately stored at −20°C. Supernatants of the digestive contents were obtained by centrifuging the microfuge tubes at 20,000 × *g* for 10 min at 4°C.

Analytical procedures. SCFA were measured by gas–liquid chromatography, after ethanolic extraction of plasma samples (14) and on aliquots of supernatants (8,000 × *g*, 5 min) of cecal contents. Bile acid analyses were performed on cecal supernatants (soluble) or after extraction. Bile acids were extracted from untreated cecal samples (total) or feces by 10 vol of ethanolic KOH (0.5M), after dispersion with a Polytron homogeneizer (Luzern, Switzerland), then heating at 80°C for one hour. For the determination of soluble bile acids, 6 vol of ethanol were added to the cecal supernatants to clarify the medium. Bile acids were measured on the supernatant (8,000 × *g*, 5 min) of the ethanolic extracts, using the reaction catalyzed by 3 α-hydroxysteroid dehydrogenase (EC 1.1.1.50; Sigma) (15).

Plasma lipoproteins were separated by density gradient ultracentrifugation (16), using pooled samples. After centrifugation in a TST 41.14 swinging-bucket rotor at 100,000 × *g* for 36 h at 15°C, the gradient was divided into 24 × 500 µL fractions and kept at 4°C for lipid analysis. Triglycerides (Biotrol, Paris, France) and total cholesterol (BioMérieux, Charbonnières-les-Bains, France) were determined in plasma and lipoprotein fractions by enzymatic procedures. The activity of HMG–CoA reductase was measured on liver microsomal fractions as described by Wilce and Kroone (17). Labelled mevalonolactone was separated from unreacted HMG–CoA by column chromatography using AG1-X8 resin (200–400 mesh, formate form; BioRad, Paris, France). Specific radioactivity of the enzyme was expressed in pmoles of [3-^{14}C]HMG–CoA transformed in [^{14}C]mevalonolactone per minute per milligram of microsomal protein, after correcting for recovery of [^{3}H]mevalonolactone from the column. The activity of FAS was determined according to the method described by Hsu *et al.* (18) on cytosolic extracts, and expressed in nmoles of [2-^{14}C]malonyl CoA incorporated into fatty acids per minute per gram of liver.

Statistical analysis. Values are given as the means ± SEM, and, where appropriate, significance of difference between mean values was determined by analysis of variance (ANOVA) coupled with the Student-Newman-Keuls multirange test. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of diets on animals and their digestive tracts. Cholestyramine in the diet had no influence on food intake and body weight gain (Table 1). Feeding diets containing 25% raw potato starch (about 18% of effectively RS) elicited a significantly higher food intake, but the final body weight was not significantly different from that of rats fed the control diet. Whatever the diets, the small intestine and liver weights were not significantly affected. In rats fed the RS diets, the large intestine (colon + cecum) was larger (+70%) than in rats fed the control diet; furthermore, there was a small but significant enlargement of the small intestine in rats fed the cholestyramine diet (+20%), compared to controls. Replacement of wheat starch by RS caused a significant drop (−14%) in the

TABLE 1
Effects of Replacement of Digestible Starch by Resistant Starch and/or Cholestyramine on Daily Food Intake, Body and Carcass Weights, and Various Cecal Parameters[a]

Diets	Daily food intake (g/d)	Body weight (g)	Small intestine wall weight (g)	Cecal + colon walls' weight (g)	Perirenal fat weight (g)	Liver weight (g)
Control	24.0 ± 0.7	284 ± 9	8.5 ± 0.5	2.58 ± 0.14	6.22 ± 0.30	12.4 ± 0.9
Cholestyramine (0.8%)	23.1 ± 1.0	283 ± 7	8.7 ± 0.6	3.10 ± 0.16[b]	6.11 ± 0.26	12.9 ± 0.7
Resistant starch (25%)	27.3 ± 0.7[b]	295 ± 7	9.5 ± 1.0	4.41 ± 0.22[c]	5.36 ± 0.20[b]	13.0 ± 0.8
Cholestyramine (0.8%) + resistant starch (25%)	26.4 ± 0.8[b]	289 ± 6	9.8 ± 0.9	4.70 ± 0.23[c]	5.28 ± 0.19[b]	12.7 ± 0.7

[a]Each value is the mean ± SEM, n = 10.
[b,c]Values within a column not sharing a superscript letter are significantly different ($P < 0.05$). The effect of dietary treatment was examined by analysis of variance coupled with the Student-Newman-Keuls test.

weight of perirenal fat, while cholestyramine alone had no significant effect.

Cecal fermentation and SCFA absorption. As shown in Table 2, the total cecal weight (cecal content + cecal wall) was significantly heavier in rats adapted to the RS (about 4-fold) or cholestyramine diets (about 1.5-fold). The cecal pH was about 7.2 in rats fed diets containing digestible starch; it was markedly acidic in rats fed RS diets (5.35 and 5.60), but there was no noticeable effect of cholestyramine on this parameter. RS diets led to a marked rise in total SCFA concentration in the cecum (from 90 to about 150 mM). In parallel, the cecal pool of SCFA was considerably elevated in rats fed the RS diets (acetate 8-fold, propionate and butyrate 10-fold). It must be noted that there was a trend toward greater quantities of butyrate in the caecum in rats fed diets containing cholestyramine.

The portal vein concentrations of SCFA were grossly proportional to the cecal SCFA pool. Table 3 shows that the portal concentration of SCFA was 6- to 8-fold higher in rats fed the RS diets than in rats fed the digestible starch diet. It must be noted that propionate absorption was particularly enhanced (8-fold) with diets containing a high amount of RS, compared to the control diet.

The arterial concentration of acetate was strongly increased in rats fed RS diets (from less than 0.2 to about 0.5 mM). No detectable concentration of butyrate or propionate was found in rats fed the control or cholestyramine diets. However, in rats fed RS diets, significant amounts of propionate were present in aortic blood (about 0.05 mM), suggesting that a small percentage of absorbed propionate escaped hepatic uptake.

Effects of RS and cholestyramine on cecal bile acids and their fecal excretion. As shown in Table 4, the cecal bile acids concentration was about 5 mM in control conditions, 40% being in soluble form. Cholestyramine led to a marked accumulation of bile acids in the cecum (about 12 mM) which were almost entirely (98%) under an insoluble bound form. In rats fed the RS diet, the cecal concentration and solubility of bile acids were markedly reduced compared to control conditions. In the group fed the RS/cholestyramine diet, the cecal bile acid concentration also was low, and the concentration of soluble bile acids was particularly low (0.15 mM). The total pool of bile acids in the cecum was increased almost 4-fold by cholestyramine and less than 2-fold by RS. Cholestyramine had a dramatic effect on bile acid excretion (+300% enhanced), whereas RS was much less effective (+70%). In rats adapted to the RS/cholestyramine diet, the fecal excretion of

TABLE 2
Effects of Replacement of Digestible Starch by Resistant Starch and/or Cholestyramine on Cecal Fermentation[a]

Diets	Total cecal[b] weight (g)	Cecal pH	Cecal concentration (mmol/L)				Cecal pool (μmoles/cecum)[b]			
			Acetate	Propionate	Butyrate	Total SCFA	Acetate	Propionate	Butyrate	Total pool SCFA
Control	2.34 ± 0.10	7.24 ± 0.06	59 ± 5	22 ± 2	9 ± 1	90 ± 5	79 ± 6	30 ± 3	12 ± 2	121 ± 8
Cholestyramine (0.8%)	3.54 ± 0.19[c]	7.19 ± 0.09	45 ± 3	17 ± 1	10 ± 1	72 ± 5[c]	94 ± 6	35 ± 3	21 ± 2[c]	150 ± 10
Resistant starch (25%)	10.15 ± 0.58[d]	5.35 ± 0.10[c]	92 ± 5[c]	46 ± 3[c]	18 ± 2[c]	156 ± 7[d]	602 ± 28[c]	305 ± 24[c]	118 ± 7[d]	1025 ± 32[c]
Cholestyramine (0.8%) + resistant starch (25%)	10.75 ± 0.69[d]	5.60 ± 0.13[c]	82 ± 6[c]	38 ± 5[c]	25 ± 3[d]	145 ± 6[d]	583 ± 33[c]	277 ± 21[c]	178 ± 13[e]	1038 ± 36[c]

[a]Each value is the mean ± SEM, n = 10; SCFA, short-chain fatty acid.
[b]Data subjected to logarithmic transformation.
[c]Values within a column not sharing a superscript letter are significantly different ($P < 0.05$). The effect of dietary treatment was examined by analysis of variance coupled with the Student-Newman-Keuls test.

TABLE 3
Effects of Replacement of Digestible Starch by Resistant Starch and/or Cholestyramine on Arterial and Portal Concentrations of SCFA[a]

Diets	Artery concentration (mmol/L)				Portal vein concentration (mmol/L)[b]			
	Acetate	Propionate	Butyrate	Total SCFA	Acetate	Propionate	Butyrate	Total SCFA
Control	0.19 ± 0.02	—	—	0.19 ± 0.02	0.31 ± 0.02	0.05 ± 0.01	0.02 ± 0.00	0.38 ± 0.02
Cholestyramine (0.8%)	0.17 ± 0.02	—	—	0.17 ± 0.02	0.27 ± 0.04	0.05 ± 0.01	0.02 ± 0.00	0.34 ± 0.03
Resistant starch (25%)	0.54 ± 0.06[c]	0.04 ± 0.01	—	0.58 ± 0.05[c]	1.37 ± 0.08[c]	0.51 ± 0.02[d]	0.15 ± 0.02[c]	2.03 ± 0.12[d]
Cholestyramine (0.8%) + resistant starch (25%)	0.49 ± 0.02[c]	0.05 ± 0.01	—	0.54 ± 0.03[c]	1.22 ± 0.09[c]	0.40 ± 0.05[c]	0.19 ± 0.02[c]	1.81 ± 0.10[c]

[a]Each value is the mean + SEM, n = 10. See Table 2 for abbreviation.
[b]Data subjected to logarithmic transformation.
[c,d]Values within a column not sharing a superscript letter are significantly different ($P < 0.05$). The effect of dietary treatment was examined by analysis of variance coupled with the Student-Newman-Keuls test.

TABLE 4
Effects of Replacement of Digestible Starch by Resistant Starch and/or Cholestyramine on Cecal and Fecal Bile Acids[a]

Diets	Cecal concentration (mmol/L)			Cecal pool (μmoles/cecum)		Bile acids fecal excretion (μmoles/day)
	Total	Soluble	% of solubility	Total	Soluble	
Control	4.7 ± 0.4	1.93 ± 0.10	41	8.2 ± 0.7	2.56 ± 0.15	16 ± 1
Cholestyramine (0.8%)	11.8 ± 1.2[d]	0.19 ± 0.02[b]	2	31.8 ± 2.5[c]	0.40 ± 0.09[b]	79 ± 4[d]
Resistant starch (25%)	1.8 ± 0.2[b]	0.37 ± 0.04[c]	21	14.8 ± 1.7[b]	2.46 ± 0.20	31 ± 2[b]
Cholestyramine (0.8%)+ resistant starch (25%)	3.5 ± 0.2[c]	0.17 ± 0.02[b]	5	30.0 ± 1.9[c]	1.08 ± 0.13[c]	63 ± 3[c]

[a]Each value is the mean ± SEM, n = 10.
[b–d]Values within a column not sharing a superscript letter are significantly different ($P < 0.05$). The effect of dietary treatment was examined by analysis of variance coupled with the student-Newman-Keuls test.

bile acids was slightly lower (63 μmoles/day) than with cholestyramine alone (79 μmoles/day).

Effects of RS and cholestyramine on plasma lipids, lipoprotein profile, and on liver activities of HMG–CoA reductase and fatty acid synthase. As shown in Figure 1, rats fed a RS diet had a significantly lower plasma cholesterol (–32%) and triglyceride (–29%) concentration than control rats or rats fed the cholestyramine diet. These effects were more accentuated in rats adapted to a RS/cholestyramine diet (–40% and –34% for cholesterol and triglycerides, respectively). The liver cholesterol and triglyceride contents were unaffected by all the experimental diets. Analysis of the plasma lipoprotein profile (Fig. 2) shows that the cholesterol-

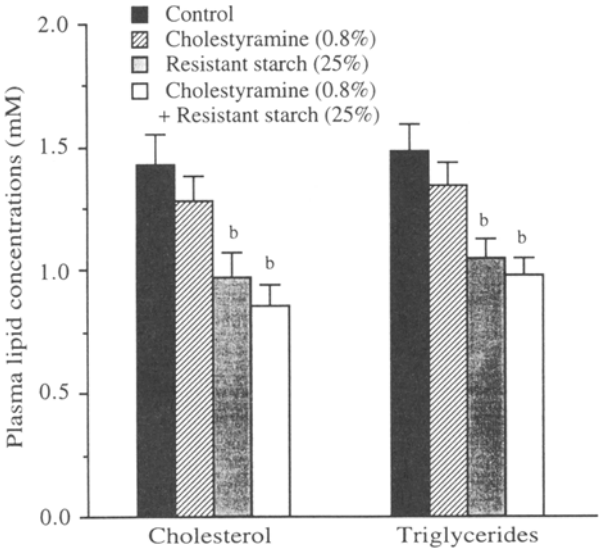


FIG. 1. Effects of replacement of digestible starch by resistant starch and/or cholestyramine on plasma cholesterol and triglycerides concentrations. Values not sharing a common letter are significantly different ($P < 0.05$).

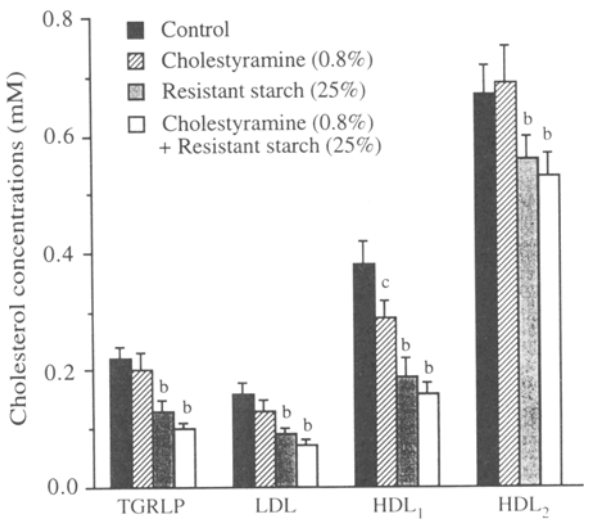


FIG. 2. Effects of replacement of digestible starch by resistant starch and/or cholestyramine on cholesterol concentration in the various lipoprotein fractions. Each value is a mean ± SEM of a triplicate analysis of a pool of ten plasmas. The triglyceride-rich lipoprotein (TGRLP) corresponded to fractions with a density lower than 1.006 kg/L. The low-density lipoprotein (LDL), high-density lipoprotein (HDL_1), and HDL_2 corresponded to fractions within a density range of 1.006–1.040, 1.040–1.080, and 1.080–1.16 kg/L, respectively. Values not sharing a common letter are significantly different ($P < 0.05$).

TABLE 5
Effects of Replacement of Digestible Starch by Resistant Starch and/or Cholestyramine on Lipid Metabolism[a]

Diets	Liver (mg/g)		Enzyme activities	
	Cholesterol	Triglycerides	HMG–CoA reductase (pmoles/min/mg protein)	Fatty acid synthase (nmoles/min/g liver)
Control	2.27 ± 0.11	10.89 ± 0.75	52 ± 8	216 ± 17
Cholestyramine (0.8%)	2.33 ± 0.14	9.35 ± 0.65	268 ± 23c	235 ± 11
Resistant starch (25%)	2.48 ± 0.10	10.48 ± 0.92	103 ± 15b	180 ± 13b
Cholestyramine (0.8%)+ resistant starch (25%)	2.56 ± 0.11	11.31 ± 0.99	223 ± 26c	154 ± 13b

[a]Each value is the mean ± SEM, n = 10. HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA.
[b,c]Values within a column not sharing a superscript letter are significantly different ($P < 0.05$). The effect of dietary treatment was examined by analysis of variance coupled with the Student-Newman-Keuls test.

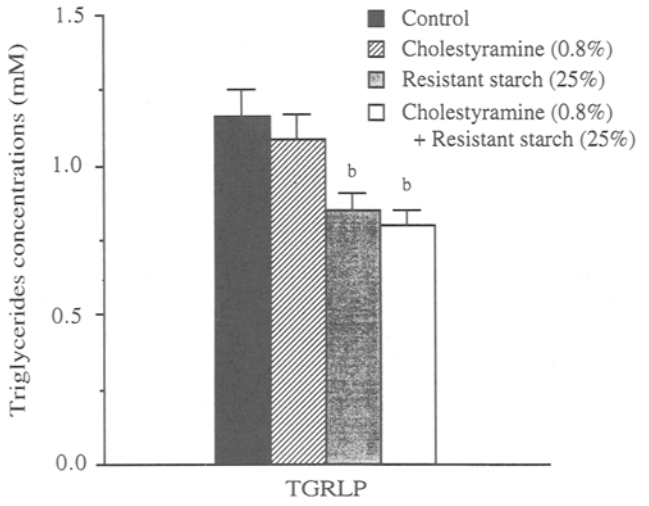


FIG. 3. Effects of replacement of digestible starch by resistant starch and/or cholestyramine on the concentration of triglycerides in the TGRLP fractions. Each value is a mean ± SEM of a triplicate analysis of a pool of ten plasmas. The TGRLP corresponded to fractions with a density lower than 1.006 kg/L. Values not sharing a common letter are significantly different ($P < 0.05$). See Figure 2 for abbreviation.

lowering effect of RS corresponded to depressed concentrations of cholesterol in all lipoprotein fractions, especially in the fraction with a density ranging from 1.040 to 1.080 [namely high-density lipoprotein (HDL_1)]. It must be noted that, with the cholestyramine diet, there was a significant decrease of cholesterol in HDL_1, which could reflect an enhanced uptake of apoE-containing particles by the liver *via* the apoB/E receptor. The concentration of triglycerides in triglyceride-rich lipoprotein (TGRLP) fractions was significantly depressed by RS (–28%) (Fig. 3). On the other hand, as shown in Table 5, the activity of HMG–CoA reductase (rate-limiting enzyme of cholesterol synthesis) was markedly increased with cholestyramine in the diet (5-fold) as well as, to a lesser extent, by RS (about 2-fold). Furthermore, the activity of FAS was negatively affected in rats fed the RS diets, especially in those fed the RS/cholestyramine diet (–30%).

DISCUSSION

It is noteworthy that RS feeding elicits a lipid-lowering effect without affecting the body weight. However, it appears that fat accumulation was slightly reduced, as shown for perirenal fat. This reduction may result from an alteration of the energetic fuels available for liver metabolism, namely a partial substitution of glucose by SCFA. Furthermore, the time course of nutrient absorption is very different when glucose is absorbed from the small intestine from digestible starch breakdown, or when SCFA are progressively produced from fermentable substrates RS, and absorbed in the large intestine. The metabolic impact of absorbed glucose is different from that of SCFA: only a small percentage of glucose is removed and utilized by the liver (19), whereas SCFA are mostly taken up by the liver and play a minor role in extrasplanchnic metabolism. This is accompanied by marked differences in the basal values and the daily fluctuations of plasma insulin (19).

RS fermentation led to a considerable increase in cecal weight (total as well as emptied), a general feature of fermentable carbohydrates in rats (6), and this is accompanied by histological changes in cecal mucosa, such as an elevated crypt height and cell number per crypt (20). The possible effects of RS on the colonic mucosa in humans are still poorly documented, but an accelerated transit rate has been reported for various fermentable carbohydrates and this tends to limit the reabsorption of bile acids (21). The microbial breakdown of RS in the large intestine may promote fermentation resulting in a large proportion of butyric acid (22), especially with a human microflora (23). However, this depends on the type of starch and associated microflora: in the present conditions (pH ≈ 5.5) in rats, high-propionic acid fermentations were rather observed with diets rich in raw potato starch. With a higher percentage of potato starch in the diet (40%), high butyric acid fermentations have been actually observed (24). It is noteworthy that cholestyramine in the diet enhanced the cecal pool of butyric acid (with an effect on the cecal concentration in rats fed a cholestyramine/RS diet). A drastic depletion of soluble bile acids in the cecum might influence the microflora and promote butyric acid production, but these changes in butyrate availability probably would have a minor role on lipid metabolism.

Previous investigations have shown that an elevated excretion of bile acids was necessary to obtain a significant cholesterol-lowering effect with fermentable fibers (25). RS can

actually increase the fecal excretion of bile acids and neutral sterols (7,13). The primary effect of RS is probably in the ileum since the cecal bile acids pool is enhanced, which reflects the extent of bile acid diversion from ileal reabsorption. Abadie *et al.* (13) have shown a comparable effect between β-cyclodextrin and potato starch. However, the effect of cyclodextrin is favored by its ring structure which is more effective than the helical structures formed by the glucose units of amylose and amylopectin (13). In this investigation, RS exhibited a better affinity for the dihydroxy bile acids than for cholic acid, in contrast to the relative affinities seen with cholestyramine.

In the cecum, bile acids are extensively deconjugated and converted to secondary bile acids by bacterial 7α-dehydroxylation (26). A passive absorption of bile acids takes place in the large intestine, but the fact that their fecal excretion is enhanced indicates that colonic absorption fails to compensate for a reduced absorption in the small intestine. Secondary bile acid absorption is likely to be depressed, because of (i) inhibition of 7-α dehydroxylase activity at acidic pH, (ii) depressed bile acid solubility due to acidic conditions. Acidic pH may also favor bile salt precipitation as insoluble calcium salts (27). Even a low concentration of cholestyramine in the diet increased bile acid output from the ileum and inhibited their passive absorption in the colon because of their insolubility (28); as a result, the fecal bile acids excretion was markedly elevated. Although RS enhanced bile acid excretion less efficiently than did cholestyramine, a marked reduction of plasma cholesterol was seen only with RS diets. Thus, this result shows that a high rate of bile acids excretion is not always sufficient to lower plasma cholesterol. In contrast, such an effect with cholestyramine has only been reported with high-fat diets supplemented with cholesterol, which could reflect some adsorption of cholesterol on the resin due to hydrophobic interaction with its apolar core (29).

In growing male rats fed on a low-fat diet, plasma cholesterol is relatively low; nevertheless, RS feeding led to a 30% lowering of plasma cholesterol. Using another type of RS, amylomaize starch, Mathé *et al.* (9) also have found a 30% reduction of plasma cholesterol but in rats (lean Zucker) with a higher basal cholesterol (about 3 mM). Morand *et al.* (19) and De Deckere *et al.* (11) have reported a marginal cholesterol-lowering effect of amylomaize starch, which suggests that potato starch might have specific effects on lipid metabolism. The effect of RS on serum cholesterol concentrations may reflect an accelerated fecal excretion of steroids (7,13) or an effect of SCFA themselves on lipid metabolism (30–32). The putative role of SCFA (chiefly propionate) on the activities of key enzymes of cholesterol metabolism is still disputed. This has been examined using various approaches: supplementation of the diet with propionate (33), intracecal infusion of propionate (34), or in isolated hepatocytes (32,35). These investigations have not always been conclusive, but it remains conceivable that propionate impairs the utilization of major lipogenic precursors (acetate, lactate) by liver cells (36,37).

In response to the elevated excretion of bile acids in rats fed RS diets, there was an induction of liver HMG–CoA reductase activity. The question arises as to whether a high availability of SCFA limits the induction of HMG–CoA reductase. Conceivably, a relatively low insulinemia in rats fed RS diets also could be a factor which limits HMG–CoA reductase induction (38). Furthermore, it appears that liver metabolism was not oriented toward lipogenesis since a low activity of FAS was observed, in accordance with previous data (39). FAS activity was not affected by cholestyramine, suggesting that this compound is very effective to accelerate cholesterogenesis but not fatty acid synthesis, as previously shown (40). Accordingly, the low percentage of cholestyramine used in this experiment failed to significantly affect plasma triglycerides, whereas induction of HMG–CoA reductase was probably sufficient to maintain the plasma cholesterol level.

With the present low-fat diets, RS was effective to lower plasma cholesterol and triglycerides. Nevertheless, a hypotriglyceridemic effect has not been systematically observed with RS (9,19); in fact, the rate of triglyceride uptake by peripheral tissues is closely dependent on the insulin status. In the present experiments, the triglyceride concentration in TGRLP was markedly depressed by the RS diet, in accordance with results obtained by Mathé *et al.* (9). The cholesterol-lowering effect of RS could be connected to a depressed secretion of very low density lipoprotein triglyceride, in keeping with the lower activity of FAS in the liver, as well as that of other enzymes involved in fatty acid synthesis (33).

In contrast to humans, most of the plasma cholesterol in rats is associated with HDL; this fraction is heterogeneous and has been separated into two major subclasses compositionally distinct with regard to the apoE content, lipid and protein composition, and particle size (41,42). RS was effective to depress cholesterol in all the liproprotein fractions, especially in HDL_1. A lowering of this apoE-containing fraction could be explained by its accelerated removal (*via* the liver apo B/E receptor) and/or its reduced formation. The first hypothesis is consistent with a high degree of HMG–CoA induction since a coordinate regulation of HMG–CoA reductase and of the apoB/E receptor have been shown at the transcriptional level (43). However, other studies have proposed that hepatic sterol synthesis and receptor-mediated uptake of low-density lipoprotein particles could be independently regulated in rats (44), thus accounting for the moderate lowering of HDL_1 cholesterol in the presence of highly induced HMG–CoA reductase in rats fed cholestyramine alone. The decrease in HDL_1 also could be explained by a reduced rate of formation, corresponding to a depressed availability of the surface components of TGRLP (45). Low levels of TGRLP were actually observed, which supports the above hypothesis. When both RS and cholestyramine were present in the diet, there was no noticeable synergism between both compounds, which suggests that the cholesterol- lowering effect of RS is not limited by its capacity to enhance bile acids excretion.

In conclusion, this study indicates that RS have hypolipidemic effects which are comparable to those of the most ef-

fective fibers. Diets containing fermentable complex carbohydrates, such as RS, are generally well-tolerated in humans although some cases of intolerance have been reported (46). Cholestyramine therapy also may be associated with troublesome side effects; thus, it remains to assess in clinical trials in which conditions RS may be used in humans, alone or in combination with cholestyramine. The lipid-lowering effects of RS were seen despite its having less effect on bile acid excretion than does cholestyramine, and the role of digestive fermentation in altering hepatic cholesterol metabolism should be reevaluated. Further study should, therefore,be made of the use of RS in the treatment of hyperlipidemia, particularly in hypertriglyceridemic subjects or those with type II diabetes.

REFERENCES

1. Stedronsky, E.R. (1994) *Biochim. Biophys. Acta 1210*, 255–287.
2. Anderson, I.H., Levine, A.S., and Lewitt, M.D. (1981) *N. Engl. J. Med. 304*, 891–892.
3. Englyst, H.N., and Cummings, J.H. (1985) *Am. J. Clin. Nutr. 42*, 778–787.
4. Flourie, B., Leblond, A., Florent, C., Rautureau, M., Bisalli, A., and Rambaud, J.C. (1988) *Gastroenterology 95*, 356–363.
5. Carroll, K.K., Hamilton, R.M.G., Huff, M.W., and Falloner, A.D. (1978) *Am. J. Clin. Nutr. 31*, S203–S207.
6. Demigné, C., and Rémésy, C. (1982) *J. Nutr. 112*, 2227–2234.
7. Sacquet, E., Leprince, C., and Riottot, M. (1983) *Reprod. Nutr. Dév. 23*, 783–792.
8. Asp, N.G., Björck, I., Holm, J., Nyman, M., and Siljeström, M. (1987) *Scand. J. Gastroenterol. 22*, 29–32.
9. Mathé, D., Riottot, M., Rostaqui, N., Sacquet, E., Navarro, N., Lécuyer, B., and Lutton, C. (1993) *J. Clin. Biochem. Nutr. 14*, 17–24.
10. Behall, K.M., Scholfield, D.J., Yuhaniak, I., and Canary J. (1989) *Am. J. Clin. Nutr. 49*, 337–344.
11. De Deckere, E.A.M., Kloots, W.J., and Van Amelsvoort, J.M.M. (1993) *J. Nutr. 123*, 2142–2151.
12. Würsch, P. (1989) in *Nutritional Value of Cereal Products, Beans and Starches* (Bourne, G.H., ed.) pp. 199–256, Karger, Amsterdam, The Netherlands.
13. Abadie, C., Hug, M., Kübli, C., and Gains, N. (1994) *Biochem. J. 299*, 725–730.
14. Rémésy, C., and Demigné, C. (1974) *Biochem. J. 141*, 85–91.
15. Turley, S.D., and Dietschy, J.M. (1978) *J. Lipid Res. 19*, 924–928.
16. Sérougne, C., Férézou, J., and Rukaj, A. (1987) *Biochim. Biophys. Acta. 921*, 522–530.
17. Wilce, P.A., and Kroone, P.A. (1992) in *Lipoprotein Analysis, A Pratical Approach* (Converse, C.A., Skinner, E.R., eds.) pp. 203–214, Oxford University Press, United Kingdom.
18. Hsu, R.Y., Butterworth, P.H.W., and Porter, J.W. (1969) in *Methods Enzymol* (Lowenstein, J.M., ed.) Vol. 14, pp. 230–235, Academic Press, New York.
19. Morand, C., Rémésy, C., Levrat, M.A., and Demigné, C. (1992) *J. Nutr. 122*, 345–354.
20. Rémésy, C., Levrat, M-A., Gamet, L., and Demigné, C. (1993) *Am. J. Physiol. 264*, G855–G862.
21. Nagengast, F.M. (1992) in *Dietary Fibre—A Component of Food* (Schweitzer, T.F. and Edwards, C.A., eds.) pp. 217–232, Springer, London.
22. MacFarlane, G.T., and Cummings, J.H. (1991) in *The Large Intestine: Physiology, Pathophysiology and Disease* (Phillips, S.F., Pemberton, J.H., and Shorter, R.G., eds.) pp. 51–92, Raven Press, New York.
23. McBurney, M.L., Cuff, D.J., and Thompson, L.U. (1990) *J. Sci. Food Agric. 50*, 79–88.
24. Levrat, M.A., Rémésy, C., and Demigné, C. (1991) *J. Nutr. Biochem. 2*, 31-36.
25. Moundras, C., Behr, S.R., Demigné, C., Mazur, A., and Rémésy, C. (1994) *J. Nutr. 124*, 2179–2188.
26. White, B.A., Caccapuoti, A.F., Fricke, R., Whitehead, T., Moshbach, E., and Hylemon, P.B. (1981) *J. Lipid Res. 22*, 891–898.
27. Younes, H., Levrat, M.A., Demigné, C., and Rémésy, C. (1993) *Ann. Nutr. Metab. 37*, 311–319.
28. Grundy, S.M., Ahrens, E.H., and Salen, L.G. (1971) *J. Lab. Clin. Med. 78*, 94–121.
29. Vahouny, G.V., Roy, T., Gallo, L.L., Story, J.A., Kritchevsky, D., and Cassidy, M. (1980) *Am. J. Clin. Nutr. 33*, 2182–2191.
30. Reddy, B.S., Watanabe, K., and Sheinfil, A. (1980) *J. Nutr. 110*, 1247–1247.
31 Vahouny, G.V., Satchithanandam, S., Chen, I., Tepper, S.A., Kritchevsky, D., Lightfoot, F.G., and Cassidy, M.M. (1988) *Am. J. Clin. Nutr. 47*, 201–206.
32. Wright, R.S., Anderson, J.W., and Bridges, S.R. (1990) *Proc. Exp. Biol. Med. 195*, 26–29.
33. Illman, R.J., Topping, D.L., McIntosh, G.H., Trimble, R.P., Storer, G.B., Taylor, M.N., and Cheng, B.Q. (1988) *Ann. Nutr. Metab. 32*, 97–107.
34. Beaulieu, K.E., and McBurney, M.I. (1992) *J. Nutr. 122*, 241–245.
35. Nishina, P.M., and Freedland, R.A. (1990) *J. Nutr. 120*, 668–673.
36. Morand, C., Besson, C., Demigné, C., and Rémésy, C. (1994) *Arch. Biochem. Biophys. 309*, 254–260.
37. Demigné, C., Morand, C., Levrat, M.A., Besson, C., Moundras, C., and Rémésy, C. (1995) *Br. J. Nutr. 74* (in press).
38. Easom, R.A., and Zammit, V.A. (1985) *Biochem. J. 230*, 747–752.
39. Morand, C., Levrat, M.A., Besson, C., Demigné, C., and Rémésy, C. (1994) *J. Nutr. Biochem. 5*, 138–144.
40. Haave, N.C., and Innis, S.M. (1991) *J. Nutr. 121*, 1529–1535.
41. Lusk, L.T., Walker, L.F., Dubien, L.H., and Getz, G.S. (1979) *Biochem. J. 183*, 83–90.
42. Lee, C.C., and Koo, S.I. (1988) *Atherosclerosis 70*, 205–215.
43. Rudling, M. (1992) *J. Lip. Res. 33*, 493–501.
44. Spady, D.K., Turley, S.D., and Dietschy, J.M. (1985) *J. Lipid Res. 26*, 465–472.
45. Eisenberg, S. (1980) *Ann. N. Y. Acad. Sci. 348*, 30–47.
46. Annison, G., and Topping, D.L. (1994) *Ann. Rev. Nutr. 14*, 298–320.

[Received February 13, 1995, and in final revised form June 16, 1995; Revision accepted July 12, 1995]

Hydrophilic Bile Acids: Prevention and Dissolution Experiments in Two Animal Models of Cholesterol Cholelithiasis

Bertram I. Cohen*, Takahiro Mikami[1], Nariman Ayyad, Akira Ohshima, R. Infante[2] and Erwin H. Mosbach

Departments of Surgery, Beth Israel Medical Center, New York, New York, and the Albert Einstein College of Medicine, The Bronx, New York

ABSTRACT: The effects of β-muricholic acid and hyocholic acid on cholesterol cholelithiasis were examined in two animal models. The following experiments were carried out: A) In a gallstone prevention study, prairie dogs were fed the lithogenic diet with or without 0.1% β-muricholic or 0.1% hyocholic acid for eight weeks. B) In a second prevention study, hamsters were fed the lithogenic diet with or without 0.1% β-muricholic acid or 0.1% hyocholic acid for six weeks. C) In a gallstone dissolution study, hamsters were fed the lithogenic diet for six weeks to induce stones; stone dissolution was examined during administration of a cholesterol-free purified diet with or without 0.1% β-muricholic acid or 0.1% hyocholic acid. In the prevention study in prairie dogs (A), both bile acids failed to prevent stone formation, the cholesterol saturation index of bile was 0.89 in the lithogenic controls, remained unchanged with hyocholic acid and increased to 1.52 in the β-muricholic acid group. In the prevention study in hamsters (B), β-muricholic acid completely inhibited the cholesterol cholelithiasis (0% stone incidence); the cholesterol saturation index of bile was 1.78 (compared to lithogenic controls, 1.37). Hyocholic acid reduced stone incidence to 16% with a cholesterol saturation index of 0.98. In the dissolution study in hamsters (C), preexisting cholesterol gallstones were not dissolved by either hydrophilic bile acid after feeding these bile acids for an additional six weeks; at the end of the experiment, the cholesterol saturation indices were below unity. These studies suggest that, in the hamster animal model, hydrophilic bile acids may be useful for the prevention of gallstones but not dissolution of preestablished cholesterol gallstones.

Lipids 30, 855–861 (1995).

The administration of exogenous bile acids to modify the natural bile acid pool and dissolve cholesterol gallstones has been in use for some 20 yr (1–4). So far this has been one of the most successful nonsurgical treatments of cholesterol cholelithiasis. Chenodeoxycholic acid and ursodeoxycholic acid were the bile acids used either alone or in combination to dissolve cholesterol stones (5–7). The mode of action of these compounds is quite different: chenodeoxycholic acid, a hydrophobic bile acid, dissolves cholesterol stones by transporting cholesterol into mixed micelles (8,9), while ursodeoxycholic acid, a hydrophilic bile acid, can dissolve cholesterol gallstones *via* (i) transfer of cholesterol from stone to micelle or (ii) formation of multilamellar vesicles, rich in cholesterol and phospholipid but low in bile acid (8,9). Ursodeoxycholic acid is safe but its use has two disadvantages: (i) enrichment of the biliary bile acid pool usually does not exceed 50% (7,8) and (ii) the intestinal bacteria can transform ursodeoxycholic acid (as well as chenodeoxycholic acid) to a hepatotoxic bile acid, lithocholic acid (10,11).

It would be desirable to use cholelitholytic bile acids which cannot be metabolized to a hepatotoxic compound. Potential candidates are bile acids substituted with a hydroxy group at carbons 3 and 6. We have previously examined the cholelitholytic properties of such compounds including hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid) and murideoxycholic acid (3α,6β-dihydroxy-5β-cholanoic acid). These compounds were very successful in preventing cholesterol gallstones (12,13). The effectiveness of trihydroxy bile acids in the treatment of cholesterol cholelithiasis remained relatively unexplored. Two compounds of interest are β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) and hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid). The trihydroxy bile acid, β-muricholic acid, was not 7-dehydroxylated by intestinal microorganisms, suggesting that it might be useful in humans in lower doses than those of the cholelitholytic bile acids currently in use (14–16). Our laboratory has studied the metabolism of β-muricholic acid in hamsters; we found that this bile acid was extracted by the liver after intravenous injection and secreted into the bile quantitatively, either as the free acid or conjugated with glycine and taurine (17). *In vitro* studies have demonstrated that β-muricholic acid can dissolve cholesterol gallstones *via* formation of a liquid crystalline mesophase (14). A second trihydroxy bile acid, hyocholic acid, was examined *in vivo* using the mouse model of cholesterol cholelithiasis; this bile acid prevented and dissolved cholesterol crystals (18). The present study examined

*To whom correspondence should be addressed at Department of Surgery, Beth Israel Medical Center, First Avenue at 16th St., New York, NY 10003.
[1]Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima 734, Japan.
[2]Inserm U.55, Hôpital Saint-Antoine, Paris, France.

Abbreviations: CSI, cholesterol saturation index; GLC, gas–liquid chromatography; LD, lithogenic diet; LI, lithogenic index; PD, purified diet.

Copyright © 1995 by AOCS Press

the efficacy of β-muricholic acid and hyocholic acid for prevention of cholesterol gallstones in hamsters and prairie dogs. These trihydroxy hydrophilic bile acids were also tested for gallstone dissolution in the hamster model.

MATERIALS AND METHODS

Bile acids. β-Muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) was a gift of the Roussel UCLAF Society (Paris, France) and had been synthesized by established methods (19–21). Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid) was obtained from Canada Packers (Toronto, Canada). Each bile acid was found to be greater than 98% pure by gas–liquid chromatographic (GLC) analyses of the trimethylsilyl ether methyl esters. 5α-Cholestane was obtained from Alltech (Deerfield, IL) and was used as internal standard for the GLC analyses of cholesterol in liver, plasma, and bile. 3α,7α-Dihydroxy-12-keto-5β-cholanoate was used as an internal standard for quantitation of the biliary bile acids.

Animals. Twenty-seven prairie dogs (*Cynomys ludovicianus*) were obtained from R-Z00 (Neshkoro, WI). The animals weighed between 800–880 g and were quarantined upon arrival for a period of two weeks; during this period, animals were given chow and water *ad libitum*. The animals were divided into three groups and fed the following diets for eight weeks: group 1 (10 animals), lithogenic diet (LD); group 2 (10 animals), LD + 0.1% hyocholic acid; group 3 (7 animals), LD + 0.1% β-muricholic acid. The diets were color-coded for identification during each feeding period. The components of the purified diet (PD) were 43.7% corn starch, 20.0% casein, 14.6% dyetrose (soluble starch), 10.0% fiber (cellulose), 5.0% salt mix (modified U.S.P. XIV salt mix, no. 200951), 4.0% butterfat, 2.0% corn oil, 0.5% vitamin mix (no. 300000), 0.2% choline chloride. The purified LD contained 0.3% cholesterol (added at the expense of corn starch). The bile acids also were added at the expense of corn starch. All diets were prepared by Dyets, Inc. (Bethlehem, PA). The food was weighed every two days to determine the daily food intake.

Male golden Syrian hamsters (*Mesocricetus auratus*) weighing between 50–70 g were purchased from Sasco Inc. (Omaha, NE). Hamsters from this supplier readily developed cholesterol gallstones when fed our LD (13). Animals were quarantined for one week; they were fed Purina Chow and water *ad libitum*. Two experiments were carried out as follows: Prevention—fifty-five hamsters were divided into three groups and fed the following diets for six weeks: group 4 (20 animals), LD; group 5 (19 animals), LD + 0.1% hyocholic acid; group 6 (16 animals), LD + 0.1% β-muricholic acid. The composition of the LD was identical to the one described above for the prairie dog.

In the dissolution study, 72 hamsters weighing between 60-80 g were fed the LD for six weeks. Fifteen animals were sacrificed to determine stone incidence (group 7a). The remaining animals were divided into three groups and fed purified regression diets (LD without cholesterol) as follows: group 7b (28 animals), PD; group 7c (14 animals), PD + 0.1% hyocholic acid; group 7d (15 animals), PD + 0.1% β-muricholic acid. All regression diets were fed for six additional weeks (total experimental period = 12 wk).

Hamsters and prairie dogs were fasted for 24 h prior to sacrifice to ensure that the gallbladders contained adequate quantities of bile for chemical analyses. Prairie dogs were anesthetized with 100–200 mg and hamsters with 20 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY). Blood was obtained *via* cardiac puncture for determination of plasma cholesterol. The abdominal cavity was exposed, and the gallbladder was examined visually for the presence of cholesterol stones. These usually could usually be observed through the gallbladder wall. Bile was removed using a Hamilton syringe; a drop of bile was immediately examined for the presence of cholesterol crystals using an Olympus MCHAP microscope (Olympus Corp., Lake Success, NY) equipped with polarization optics. Gallstones were crushed and analyzed for cholesterol and other components by Fourier transform infrared spectrometry using a Perkin Elmer 1710 spectrometer (Perkin Elmer, Norwalk, CT). The diameter of the stones usually ranged from 1 to 5 mm.

Analytical procedures for plasma, liver, and bile. Bile (hamster, 3 μL; prairie dog, 50 μL) from each animal was immediately centrifuged at 3000 × *g* for 30 min and aliquots were taken for analyses of the biliary lipids (22). Biliary bile acids, cholesterol, and phospholipids were analyzed using procedures described earlier (12,13). Bile acids were analyzed by GLC as the methyl ester acetates. Cholesterol in liver and plasma was determined from portions of these tissues obtained at sacrifice; quantitation of cholesterol was carried out after preparation of the trimethylsilyl ether derivative using 5α-cholestane as an internal standard. The cholesterol saturation index (SI), lithogenic index (LI) was calculated as described earlier (23,24). The calculation of the CSIs using the hydrophilic bile acid correction was made according to Carey (24). This correction applies only to total lipid concentrations between 5.0 to 15 g/dL. These corrections were made for β-muricholic acid and hyocholic acid using data for ursodeoxycholic acid (the only hydrophilic bile acid for which these equations were available).

Statistical methods and data analyses. The data are reported as mean ± SD. The differences in gallstone incidence between groups were calculated using Fisher's exact test (25). Statistical significance of other values was calculated by analyses of variance to determine the F statistic. When the F statistic was significant ($P < 0.05$), Student's *t*-test was applied to the data to determine the level of significance.

RESULTS

Hamster and prairie dog models of cholesterol cholelithiasis were examined to determine whether hydrophilic trihydroxy bile acids could prevent cholesterol gallstones. In these studies, hamsters averaged 50 g in weight, and prairie dogs averaged between 800–841 g. The hamster model also was employed to test the efficacy of hydrophilic bile acids for disso-

lution of preestablished cholesterol gallstones; hamsters averaged 72 g at the start of the induction phase (week 0) and 109–119 g at the start of the dissolution phase (week 7). All animals remained healthy and gained weight normally throughout the feeding periods. The total liver weights for either hamsters or prairie dogs fed hyocholic acid (Table 1) were slightly lower than those of the other groups.

The incidence of gallstones and crystals was determined at sacrifice (Table 2). Prevention of cholesterol stones was different in the two models. In prairie dogs (groups 1–3), gallstone prevention was not observed with either hydrophilic bile acid. In the Sasco hamster, the incidence of cholesterol stones and crystals was significantly reduced by either hyocholic acid or β-muricholic acid (16 and 0%, group 5 vs. group 6) compared to the LD (90%, group 4). The hamster model also was tested for dissolution of stones using the same hydrophilic bile acids after stones had formed. In phase 1 of this study (group 7a), cholesterol stones were present in 93% of the animals after six weeks. The diet was changed to one containing the identical components of the LD but without cholesterol—PD; this diet was used to facilitate the dissolution of established stones by bile acids. The bile acids were incorporated into the PD as follows: hyocholic acid, 0.1%, group 7c; β-muricholic acid, 0.1%, group 7d. Dissolution of established gallstones was not observed after administration of these diets for six weeks. Stone incidence in all groups ranged from 78–93%.

Table 3 summarizes the cholesterol levels in liver, plasma, and bile. In the prairie dog, both hydrophilic bile acids significantly reduced the cholesterol levels in liver and plasma compared to the LD (group 1, $P < 0.01$). Biliary cholesterol was reduced by β-muricholic acid (1.91 mg/mL) and hyocholic acid (2.91 mg/mL), but these differences were not statistically significant from the controls. In the hamster stone prevention study (groups 4–6), both hydrophilic bile acids significantly reduced liver and plasma cholesterol compared to the LD; hyocholic acid reduced the amount of biliary cholesterol (from 5.3 to 3.9 mg/mL), but this decrease was not significant. β-Muricholic acid did not reduce biliary cholesterol levels even though it completely abolished cholesterol gallstone formation. The gallstone dissolution study was carried out in two phases: induction (group 7a) and regression (groups 7b–7d). Hamsters fed the regression diets + bile acid for a period of six weeks had significantly lower levels of cholesterol in liver and plasma. Biliary cholesterol levels were reduced with 0.1% hyocholic acid in the diet (0.6 mg/mL) but not with β-muricholic acid (1.6 mg/mL). These changes in biliary cholesterol occurred in the absence of cholesterol gallstone dissolution (Table 2).

The biliary lipid composition is summarized in Table 4. In the prairie dog (groups 1–3), the mole% cholesterol in bile ranged from 2.2 (group 2, LD + hyocholic acid) to 5.8 (group 3, LD + β-muricholic acid); the incidence of cholesterol gallstones was similar in all groups. Total lipids (g/dL) were highest in prairie dogs fed LD + hyocholic acid (18.7 g/dL); this group had the lowest CSI (0.76). The highest CSI (1.52) was observed in prairie dogs given 0.1% β-muricholic acid; this group had the greatest incidence of gallstones. In the hamster stone prevention study, stone incidence correlated inversely with the CSI; animals in group 6 had the highest CSI (1.78), but gallstone incidence was 0%. Hamsters in the dissolution study had significant reductions in mole% cholesterol and CSI at the end of twelve weeks (group 7c, 0.5, and 0.11, respectively). These changes occurred in the absence of stone dissolution. Total biliary lipids were 12.2 g/dL in hamsters given the PD + hyocholic acid. This was significantly higher than in animals fed PD or PD + β-muricholic acid (6.4 and 6.3 g/dL, respectively). The CSIs for animals fed the hydrophilic bile acids also were calculated using the ursodeoxycholic acid correction described by Carey (24); these calculations could only be carried out when the total lipid concentration was between 5–15 g/dL. The CSI increased in all cases

TABLE 1
Weight, Food Intake, and Liver Weights of Animals[a]

Group/diet	Number of animals	Initial weight (g)	Final weight (g)	Liver weight (g)	Food intake (g/d)
Prairie dog—prevention					
1. Lithogenic diet (LD)	10	810 ± 45	954 ± 83	33 ± 9	40 ± 1
2. LD + 0.1% hyocholic acid	10	841 ± 83	995 ± 97	23 ± 4	39 ± 1
3. LD + 0.1% β-muricholic acid	7	800 ± 70	1060 ± 208	26 ± 7	46 ± 3
Hamster—prevention					
4. LD	20	51 ± 2	109 ± 8	6.8 ± 0.6	11 ± 1
5. LD + 0.1% hyocholic acid	19	51 ± 3	105 ± 9	5.1 ± 0.7	11 ± 1
6. LD + 0.1% β-muricholic acid	16	50 ± 2	101 ± 9	5.5 ± 0.5	10 ± 1
Hamster—dissolution[b]					
7a. LD	72	72 ± 5	109 ± 19	7.3 ± 1.6	12 ± 1
7b. Purified diet (PD)	28	119 ± 14	124 ± 15	6.4 ± 1.1	12 ± 1
7c. PD + 0.1% hyocholic acid	14	116 ± 14	113 ± 10	4.7 ± 0.4	11 ± 1
7d. PD + 0.1% β-muricholic acid	15	109 ± 19	111 ± 25	6.1 ± 2.0	11 ± 1

[a]See the Materials and Methods section for composition of diets; numbers are average ± SD; PD contains no cholesterol.
[b]72 Animals in group 7a fed LD for six weeks; fifteen animals were sacrificed and remainder of animals were divided into three groups (7b–7d) and fed PD with and without bile acids for an additional six weeks.

TABLE 2
Gallstone Incidence at Sacrifice[a]

Group/diet	Cholesterol gallstones	Cholesterol crystal
Prairie dog—prevention		
1. LD	10/10	10/10
2. LD + 0.1% hyocholic acid	9/10	9/10
3. LD + 0.1% β-muricholic acid	6/7	7/7
Hamster—prevention		
4. LD	18/20	19/20
5. LD + 0.1% hyocholic acid	3/19[b]	6/19[b]
6. LD + 0.1% β-muricholic acid	0/16[b]	0/16[b]
Hamster—dissolution study		
7a. LD	14/15	14/15
7b. PD	26/28	26/28
7c. PD + 0.1% hyocholic acid	11/14	11/14
7d. PD + 0.1% β-muricholic acid	13/15	13/15

[a]See the Materials and Methods section for details. See Table 1 for abbreviations. The information with the slash marks indicates number of gallstones or crystals per number of animals examined (e.g., 9/10 indicates the number of animals with gallstones or crystals per number of animals examined).
[b]Differs from group 4, $P < 0.01$ by Fisher's exact test.

TABLE 3
Cholesterol Levels in Tissues of Hamsters and Prairie Dogs[a]

Group/diet	Liver (mg/g)	Plasma (mg/dL)	Bile (mg/mL)
Prairie dog—prevention			
1. LD	9.7 ± 5.6	494 ± 302	3.3 ± 1.7
2. LD + 0.1% hyocholic acid	2.6 ± 0.9[b]	159 ± 68[b]	2.9 ± 1.3
3. LD + 0.1% β-muricholic acid	3.6 ± 2.0[b]	221 ± 87[b]	1.9 ± 2.1
Hamster—prevention			
4. LD	100.3 ± 16.6	331 ± 35	5.3 ± 1.2
5. LD + 0.1% hyocholic acid	27.9 ± 11.5[c]	208 ± 28[c]	3.9 ± 1.1
6. LD + 0.1% β-muricholic acid	67.9 ± 11.6[c]	252 ± 25[c]	5.5 ± 1.7
Hamster—dissolution			
7a. LD	50.8 ± 6.8	308 ± 63	4.5 ± 1.4[d]
7b. PD	29.9 ± 11.1	284 ± 45	1.0 ± 0.7
7c. PD + 0.1% hyocholic acid	3.6 ± 1.1[e]	96 ± 24[e]	0.6 ± 0.4
7d. PD + 0.1% β-muricholic acid	19.2 ± 13.4	186 ± 74	1.6 ± 0.9

[a]See the Materials and Methods section for analytical procedures. See Table 1 for abbreviations.
[b]Differs significantly from group 1, $P < 0.01$ by *t*-test.
[c]Differs significantly from group 4, $P < 0.01$.
[d]Differs significantly from groups 7b–7d, $P < 0.01$.
[e]Differs significantly from groups 7b and 7d, $P < 0.01$.

where the correction was applied. Presumably, the modified CSI for prairie dogs fed 0.1% hyocholic acid also would be above 1.0 if the bile acid correction were applicable.

The biliary bile acid composition is summarized in Table 5. Feeding hyocholic acid to prairie dogs resulted in a significant enrichment of this bile acid (11.9%) and its metabolite (murideoxycholic acid, 22.7%) in bile. Feeding hyocholic acid to the hamster resulted in a much smaller enrichment of this bile acid in bile (prevention study, group 5, 0.7%; dissolution study, group 7c, 3.6%). A significant portion of the hyocholic acid was 7-dehydroxylated to hyodeoxycholic acid (Sigma, St. Louis, MO) (prevention study, group 5, 46.8%; dissolution study, group 7c, 52.6%). Administration of β-muricholic acid (a bile acid not normally detected in prairie dog or hamster bile) resulted in an increase in the amount of this bile acid in all groups (group 3, 38.8%; group 6, 16.4%, and group 7d, 17.7%).

DISCUSSION

Interest in medical therapies of cholesterol cholelithiasis has recently been overshadowed by the development of laparoscopic cholecystectomy (26). Nevertheless, prevention of stone development and/or stone recurrence by pharmacologic or dietary means remains an important goal. Long-term bile acid therapy has been suggested to attain this objective, but this presents problems of cost and poor compliance. If cheaper, longer-acting bile acids should become available, ad-

TABLE 4
Biliary Lipids in Prairie Dogs and Hamsters[a]

Group/diet	Cholesterol (mol%)	Phospholipid (mol%)	Bile acids (mol%)	Total lipid (g/dL)	Cholesterol saturation index	Modified cholesterol saturation index
Prairie dog—prevention						
1. LD	4.1 ± 0.5	9.9 ± 2.9	86.0 ± 4.6	10.7 ± 4.3	0.89 ± 0.17	NA
2. LD + 0.1% hyocholic acid	2.2 ± 0.9[b]	2.9 ± 0.9[b]	94.9 ± 1.4	18.7 ± 5.2[c]	0.76 ± 0.33	NA
3. LD + 0.1% β-muricholic acid	5.8 ± 3.3[d]	8.5 ± 1.5	85.7 ± 4.8	7.5 ± 2.3[e]	1.52 ± 0.65[f]	2.66 ± 0.50
Hamster—prevention						
4. LD	7.1 ± 2.0	14.4 ± 2.3	78.5 ± 4.3	11.6 ± 4.2	1.37 ± 0.51	NA
5. LD + 0.1% hyocholic acid	4.3 ± 1.1[g]	10.2 ± 0.9[g]	85.5 ± 1.4[g]	12.5 ± 3.5	0.98 ± 0.29	2.13 ± 0.19
6. LD + 0.1% β-muricholic acid	9.2 ± 3.0[h]	14.3 ± 2.0	76.5 ± 3.3	8.4 ± 2.0[i]	1.78 ± 0.62[j]	2.00 ± 0.19
Hamster—dissolution						
7a. LD	4.2 ± 1.3	10.9 ± 2.2	84.3 ± 4.1	14.9 ± 7.1	0.87 ± 0.25	NA
7b. PD	1.9 ± 0.7	16.4 ± 1.4	82.6 ± 1.7	6.4 ± 2.6	0.37 ± 0.14	NA
7c. PD + 0.1% hyocholic acid	0.5 ± 0.2[k]	11.9 ± 2.5[k]	87.6 ± 2.8	12.2 ± 3.6[k]	0.11 ± 0.03[k]	0.36 ± 0.09
7d. PD + 0.1% β-muricholic acid	3.6 ± 0.9	17.7 ± 1.1	78.7 ± 0.1	6.3 ± 1.5	0.66 ± 0.18	0.76 ± 0.14

[a]See the Materials and Methods section for analytical procedures; cholesterol saturation indices with bile acid correction are calculated from the amounts of the hydrophilic bile acids in bile. NA = nonapplicable. See Table 1 for abbreviations.
[b]Differs significantly from groups 1 and 3, $P < 0.01$.
[c]Differs significantly from groups 1 and 3, $P < 0.01$.
[d]Differs significantly from group 2, $P < 0.03$.
[e]Differs significantly from group 1, $P < 0.05$.
[f]Differs significantly from groups 1 and 2, $P < 0.01$.
[g]Differs significantly from groups 4 and 6, $P < 0.01$.
[h]Differs significantly from group 5, $P < 0.02$.
[i]Differs significantly from groups 4 and 5, $P < 0.01$.
[j]Differs significantly from group 5, $P < 0.01$.
[k]Differs significantly from groups 7b and 7d, $P < 0.01$.

ministration of such compounds for stone prevention might eventually be feasible.

Hydrophilic bile acids solubilize cholesterol in liquid crystals rather than in micelles, thus permitting bile to become supersaturated with cholesterol without the rapid formation of cholesterol monohydrate crystals (27–29). The present studies examined the cholelitholytic properties of two hydrophilic trihydroxy bile acids (hyocholic acid and β-muricholic acid), in two animal models. We studied (i) cholesterol gallstone prevention in the prairie dog and the hamster and (ii) dissolution of established gallstones in the hamster. Previous work had shown that other hydrophilic bile acids, hyodeoxycholic acid, and murideoxycholic acid prevented cholesterol stones in the prairie dog and in the hamster (12,13). Hyodeoxycholic acid was only marginally successful in dissolving cholesterol gallstones in the prairie dog (11). Cholesterol stones dissolved in the presence of supersaturated bile, suggesting that cholesterol was solubilized in liquid crystalline vesicles (27–30). Hyodeoxycholic acid is not useful in humans because it is glucuronidated and lost from the enterohepatic circulation *via* urinary excretion (15). Murideoxycholic acid likewise must be used with great caution because in the presence of cholesterol stones it precipitates as the insoluble calcium/sodium salt (31).

There is some prior evidence that hyocholic and β-muricholic acids may be potentially useful as litholytic agents. Hyocholic acid dissolved cholesterol gallstones in a mouse model of cholesterol cholelithiasis; the stones dissolved almost completely with the addition of 0.1 or 0.3% hyocholic acid to a standard pellet diet (18). The mouse model is probably less suitable to study the preventive effects of hydrophilic bile acids since stone induction requires large amounts of dietary cholesterol (1.0%) plus cholic acid (0.5%) (32,33). The presence of cholic acid confounds the interpretation of results produced by added hydrophilic bile acids. β-Muricholic acid dissolved cholesterol gallstones *in vitro* at physiological concentrations *via* the formation of multilamellar liquid crystals (14).

The effectiveness of the hydrophilic bile acids for stone prevention was different in our two animal models. In the Sasco hamster, both hyocholic acid and β-muricholic acid prevented the formation of cholesterol gallstones, while in the prairie dog they were ineffective. This result is difficult to understand in terms of the observed liver and plasma cholesterol concentrations; both hydrophilic bile acids significantly reduced the accumulation of the exogenous cholesterol in liver and plasma. Hydrophilic bile acids were reported to reduce cholesterol absorption from the gut because they were poor

TABLE 5
Biliary Bile Acid Composition at Sacrifice[a]

Group/diet	Total bile acid (mg/mL)	LCA (%)	DCA (%)	CDCA (%)	CA (%)	HDCA (%)	HCA (%)	β-MCA (%)	MDCA (%)	Other (%)
Prairie dog—prevention										
1. LD	69.5 ± 25.3	0.6 ± 0.2	2.0 ± 1.1	32.2 ± 13.7	56.3 ± 10.6	0.3 ± 0.1	—	—	0.6 ± 0.4	8.0 ± 1.2
2. LD + 0.1% hyocholic acid	143.3 ± 41.2[b]	0.2 ± 0.1	1.8 ± 0.9	4.7 ± 1.7	25.4 ± 9.3	26.1 ± 7.3	11.9 ± 4.0	—	22.7 ± 7.1	8.0 ± 0.8
3. LD + 0.1% β-muricholic acid	46.7 ± 13.9	0.8 ± 0.4	0.8 ± 0.4	10.5 ± 5.1	26.1 ± 5.9	1.8 ± 0.5	—	38.8 ± 5.2	1.0 ± 0.4	19.4 ± 3.2
Hamster—prevention										
4. LD	65.3 ± 22.9	4.3 ± 1.6	11.8 ± 2.1	26.5 ± 3.7	29.5 ± 4.5	10.4 ± 3.7	—	—	3.2 ± 0.9	14.3 ± 1.2
5. LD + 0.1% hyocholic acid	83.2 ± 23.2[c]	2.3 ± 0.5	9.8 ± 2.0	8.3 ± 1.9	16.3 ± 2.5	46.8 ± 3.9[c]	0.7 ± 0.2	—	11.3 ± 2.5[c]	4.5 ± 0.4
6. LD + 0.1% β-muricholic acid	49.2 ± 10.4	3.8 ± 0.7	13.0 ± 3.1	18.6 ± 3.6	24.1 ± 2.2	14.6 ± 4.0	—	16.4 ± 3.5	6.8 ± 2.8	2.7 ± 0.3
Hamster—dissolution										
7a. LD	98.9 ± 51.1	5.2 ± 0.8	12.6 ± 2.8	38.4 ± 4.2	23.4 ± 5.4	12.0 ± 3.1	—	—	3.4 ± 1.7	3.8 ± 1.7
7b. PD	37.8 ± 16.2	2.9 ± 0.3	14.8 ± 3.1	22.1 ± 5.4	36.3 ± 4.7	13.9 ± 4.4	—	—	3.6 ± 0.8	20.3 ± 1.4
7c. PD + 0.1% hyocholic acid	81.8 ± 27.3[d]	0.9 ± 0.3	5.0 ± 1.0	5.8 ± 3.0	11.3 ± 1.0	52.6 ± 2.3	3.6 ± 3.1	—	13.1 ± 5.1	7.7 ± 1.1
7d. PD + 0.1% β-muricholic acid	42.7 ± 20.6	1.5 ± 0.9	7.7 ± 2.8	19.3 ± 7.3	23.4 ± 3.6	7.4 ± 7.2	—	17.7 ± 2.0	3.8 ± 3.2	19.2 ± 7.1

[a]See the Methods and Materials section for details on bile acid analyses; "other" includes allocholic acid and keto bile acids. LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; HCA, hyocholic acid; β-MCA, β-muricholic acid; MDCA, murideoxycholic acid. See Table 1 for other abbreviations.

[b]Differs significantly from groups 1 and 3, $P < 0.01$.

[c]Differs significantly from groups 4 and 6, $P < 0.01$.

[d]Differs significantly from groups 7b and 7d, $P < 0.01$.

micelle formers (34). A previous study in hamsters using another hydrophilic bile acid (3α,6β-dihydroxy-6α-methyl-5β-cholanoic acid) indicated that gallstones can be completely prevented (0/11) even though biliary bile acid enrichment was quite low (enrichment of fed bile acid = 4.1%) (13). It thus appears that the percentage of enrichment of the administered bile acid does not necessarily correlate with gallstone prevention.

In a previous study in the prairie dog, the hydrophilic bile acid, hyodeoxycholic acid, prevented cholesterol cholelithiasis (12). We anticipated that β-muricholic acid and hyocholic acid would behave similarly in this model. However, this may not have taken place because the amounts (mg/mL) of 3,6-dihydroxy, hydrophilic bile acids were not sufficiently elevated with either hyocholic acid (hyodeoxycholic acid + murideoxycholic acid = 51%) and with β-muricholic acid (hyodeoxycholic acid + murideoxycholic acid = 41.6%) (12). The present study is not strictly comparable to previous work with 6-hydroxy bile acids in the prairie dog (12). In the earlier experiments, the LD was prepared with soy protein and contained no butterfat. The diet used in the present study was identical for prairie dogs and hamsters and contained butterfat and casein (in place of soy protein). It seems possible that the butterfat/casein diet provided an enhanced lithogenic stimulus which reduced the cholelitholytic effect of the exogenous bile acids.

Dissolution of preestablished cholesterol gallstones was studied in the hamster. Spontaneous dissolution did not occur in six weeks when a cholesterol-free, PD was substituted for the LD. Addition of 0.1% of the hydrophilic bile acids, hyocholic acid, or β-muricholic acid to the diet did not induce dissolution. In all of the regression groups (7b–d), the LIs of bile were less than unity with the greatest decrease observed in hamsters fed hyocholic acid. The failure of the cholesterol stones to dissolve in the presence of unsaturated bile was unexpected. A combination of longer regression times (twelve weeks) plus administration of increased doses of bile acid might give better results, but this was not attempted in the present study because of the limited amounts of bile acids available. Presumably, the stones did not dissolve because the rate of equilibration between the biliary cholesterol monohydrate crystals and the desaturated bile was too slow. Other factors which might also be involved include gallbladder motility, mucin production, and nucleating proteins.

In conclusion, it was demonstrated that certain hydrophilic bile acids, namely hyocholic acid and β-muricholic acid, prevent gallstones in the hamster model, but not in the prairie dog model of cholesterol cholelithiasis. Dissolution of established cholesterol stones was not achieved in the hamster model.

ACKNOWLEDGMENTS

This work was supported in part by USPHS grants R01 DK-43204 from the National Institute of Diabetes and Digestive and Kidney Disease (BIC), R37 HL-24061 from the National Heart, Lung, and Blood Institute (EHM), and a grant from the Singer-Hellman Fund.

REFERENCES

1. Danzinger, R.G., Hofmann, A.F., Schoenfield, L.J., and Thistle, J.L. (1972) Dissolution of Cholesterol Gallstones by Chenodeoxycholic Acid, *N. Engl. J. Med. 286*, 1–8.
2. Nakagawa, S., Makino, I., Ishizaki, T., and Dohi, I. (1977) Dissolution of Cholesterol Gallstones by Ursodeoxycholic Acid, *Lancet 2*, 367–369.
3. Northfield, T.C., Jazrawi, R.P., and Petroni, M.L. (1990) Medical Dissolution Therapy, in *Strategies for the Treatment of Hepatobiliary Diseases* (Paumgartner, G., Stiehl, A., Barbara, L., and Roda, E., eds.) pp. 163–184, Kluwer Academic Publishers, The Netherlands.
4. Fromm, H.M., and Malavolti, M. (1988) Dissolving Gallstones, *Adv. Intern. Med. 33*, 409–430.
5. Hofmann, A.F., Thistle, J.L., Klein, P.D., Szczepanski, P.A., and Yu, P.Y.S. (1978) Chenotherapy for Gallstone Dissolution. II. Induced Changes in Bile Composition and Gallstone Response, *JAMA 239*, 1138–1144.
6. Fisher, M.M., Roberts, E.A., Rosen, I.E., Shapero, T.F., Sutherland, L.R., Davies, R.S., Bacchus, R., and Lee, S.V. (1985) The Sunnybrook Gallstone Study: A Double-Blind Controlled Trial of Chenodeoxycholic Acid for Gallstone Dissolution, *Hepatology 5*, 102–107.
7. Carulli, N.M., Ponz de Leon, M., Zironi, F., Pinetti, A., Smerieri, A., Iori, R., and Loria, P. (1980) Hepatic Cholesterol and Bile Acid Metabolism in Subjects with Gallstones: Comparative Effects of Short-Term Feeding of Chenodeoxycholic Acid and Ursodeoxycholic Acid, *J. Lipid Res. 21*, 35–43.
8. Hofmann, A.F., (1984) Medical Treatment of Cholesterol Gallstones by Bile Desaturating Agents, *Hepatology 4*, 199S–208S.
9. Salvioli, G., Igimi, H., and Carey, M.C. (1984) Cholesterol Gallstone Dissolution in Bile: Dissolution Kinetics of Crystalline Cholesterol Monohydrate by Conjugated Chenodeoxycholate–Lecithin and Conjugated Ursodeoxycholate–Lecithin Mixtures—Dissimilar Phase Equilibria and Dissolution Mechanisms, *J. Lipid Res. 24*, 701–720.
10. Miyai, K., Javitt, N.B., Gochman, N., Jones, H.M., and Baker, D. (1982) Hepatotoxicity of Bile Acids in Rabbits, *Lab. Invest. 46*, 428–437.
11. Bazzoli, F., Fromm, H., Sarva, R.P., Sembrat, R.F., and Ceryak, S. (1982) Comparative Formation of Lithocholic Acid from Chenodeoxycholic Acid and Ursodeoxycholic Acid in the Colon, *Gastroenterology 83*, 753–760.
12. Singhal, A.K., Cohen, B.I., Mosbach, E.H., Une, M., Stenger, R.J., McSherry, C.K., May-Donath, P., and Palaia, T. (1984) Prevention of Cholesterol-Induced Gallstones by Hyodeoxycholic Acid in the Prairie Dog, *J. Lipid Res. 25*, 539–549.
13. Cohen, B.I., Matoba, N., Mosbach, E.H., Ayyad, N., Hakam, K., Ock Suh, S., and McSherry, C.K. (1990) Bile Acids Substituted in the 6-Position Prevent Cholesterol Gallstone Formation in the Hamster, *Gastroenterology 98*, 397–405.
14. Montet, J.C., Parquet, M., Sacquet, E., Montet, A., Infante, R., and Amic, J. (1987) β-Muricholic Acid: Potentiometric and Cholesterol-Dissolving Properties, *Biochim. Biophys. Acta 918*, 1–10.
15. Sacquet, E., Parquet, M., Riottot, M., Raizman, A., Jarrige, P., Huguet, C., and Infante, R. (1983) Intestinal Absorption, Excretion, and Biotransformation of Hyodeoxycholic Acid in Man, *J. Lipid Res.* 24, 604–613.
16. Sacquet, E., Parquet, M., Riottot, M., Raizman, A., Nordlinger, B., and Infante, R. (1985) Metabolism of β-Muricholic acid in man, *Steroids 45*, 411–426.
17. Miki, S., Mosbach, E.H., Cohen, B.I., Mikami, T., Infante, R., Ayyad, N., and McSherry, C.K. (1993) Metabolism of β-Muricholic Acid in the Hamster and Prairie Dog, *J. Lipid Res. 34*, 1709–1716.
18. Dusserre, J.P., Montet, A.M., and Montet, J.C. (1988) Effect of Hyocholic Acid on the Prevention and Dissolution of Biliary Cholesterol Crystals in Mice, *Can. J. Physiol. Pharmacol. 66*, 1028–1034.
19. Hsia, S.L. (1971) Hyocholic Acid and Muricholic Acids, in *The Bile Acids* (Nair, P.P., and Kritchevsky, D., eds.) Vol. 1, pp. 95–120, Plenum Press, New York.
20. Hsia, S.L., Matschiner, J.T., Mahowald, T.A., Elliott, W.H., Doisy, Jr., E.A., Thayer, S.A., and Doisy, E.A. (1957) Bile Acids. VI. The Structure and Synthesis of Acid II, *J. Biol. Chem. 226*, 667–671.
21. Hsia, S.L., Matschiner, J.T., Mahowald, T.A., Elliott, W.H., Doisy, Jr., E.A., Thayer, S.A., and Doisy, E.A. (1957) Bile Acids. V. Chemical Studies on New Bile Acids from the Rat and the Hog, *J. Biol. Chem. 225*, 811–823.
22. Cohen, B.I., Ayyad, N., Mikami, T., Mikami, Y., and Mosbach, E.H. (1994) Effects of Dietary Fat and Fatty Acids on Sterol Balance in Hamsters, *Lipids 29*, 503–508.
23. Kuroki, S., Cohen, B.I., Carey, M.C., and Mosbach, E.H. (1986) Rapid Computation with the Personal Computer of the Percent Cholesterol Saturation of Bile Samples, *J. Lipid Res. 27*, 442–446.
24. Carey, M.C. (1978) Critical Tables for Calculating the Cholesterol Saturation of Native Bile, *J. Lipid Res. 19*, 945–955.
25. Snedecor, G.W., and Cochran, W.G. (1946) *Statistical Methods*, pp. 56–81, The Iowa State University Press, Ames, Iowa.
26. NIH Consensus Development Panel on Gallstones and Laparoscopic Cholecystectomy (1993) Gallstones and Laparoscopic Cholecystectomy, *JAMA 269*, 1018–1024.
27. Park, Y.H., Igimi, H., and Carey, M.C. (1984) Dissolution of Human Cholesterol Gallstones in Simulated Chenodeoxycholate-Rich and Ursodeoxycholate-Rich Biles, *Gastroenterology 87*, 150–158.
28. Igimi, H., Asakawa, S., Watanabe, D., and Shimura, H. (1983) Liquid Crystal Formation in Ursodeoxycholic-Rich Human Gallbladder Bile, *Gastroenterol. Jpn.* 18, 93–97.
29. Cohen, D.E., Angelico, M., and Carey, M.C. (1990) Structural Alterations in Lecithin–Cholesterol Vesicles Following Interactions with Monomeric and Micellar Bile Salts: Physical–Chemical Basis for Subselection of Biliary Lecithin Species and Aggregative States of Biliary Lipids During Bile Formation, *J. Lipid Res. 31*, 55–70.
30. Cohen, B.I., Mosbach, E.H., McSherry, C.K., Rzigalinski, B., and Kuroki, S. (1986) A Hydrophilic Bile Acid Effects Partial Dissolution of Cholesterol Gallstones in the Prairie Dog, *Lipids 21*, 575–579.
31. Cohen, B.I., Ayyad, N., Mosbach, E.H., McSherry, C.K., Matoba, N., Hofmann, A.F., Ton-Nu, H., Peng, Y., Schteingart, C.D., and Stenger, R.J. (1991) Replacement of Cholesterol Gallstones by Murideoxycholic Acid, *Hepatology 14*, 158–168.
32. Tepperman, J. (1965) Experimental Production of Gallstones, *Gastroenterology 48*, 261–262.
33. Tepperman, J., Caldwell, F., and Tepperman, H. (1964) Induction of Gallstones in Mice by Feeding a Cholesterol-Cholic Acid Containing Diet, *Am. J. Physiol. 206*, 628–634.
34. Westergaard, H., and Dietschy, J.M. (1976) The Mechanisms Whereby Bile Acid Micelles Increase the Rate of Fatty Acid and Cholesterol Uptake into the Intestinal Mucosa Cell, *J. Clin. Invest. 58*, 97–108.

[Received April 4, 1995; Revision accepted July 7, 1995]

Continuous Spectrophotometric Assay of Conjugated Bile Acid Hydrolase

Lyndon C. Kirby[a], Robert A. Klein[b] and James P. Coleman[a,*]

[a]Department of Microbiology and Immunology, School of Medicine and [b]Department of Chemistry, East Carolina University, Greenville, North Carolina 27858

ABSTRACT: Conjugated bile acid hydrolase (CBAH) refers to a class of enzymes which catalyze the cleavage of the amino acid moieties from conjugated bile acids. These enzymes are significant because of their role in providing substrates for further microbial metabolism in the gastrointestinal tract. They also are used in research laboratories for the deconjugation of bile acids prior to structural analyses. A continuous spectrophotometric assay for CBAH activity was developed using a conjugate of cholic acid and the chromophore, 5-amino-2-nitro-benzoic acid. The free chromophore is detected by virtue of its absorbance at 410 nm. The CBAH from *Clostridium perfringens* displayed a K_m for this substrate of 120 μM. These results demonstrate that this new compound functions as an effective substrate of the enzyme and forms the basis for a convenient and rapid method to monitor CBAH activity.
Lipids 30, 863–867 (1995).

Mammalian bile acids are C-24 steroid carboxylic acid molecules which are synthesized in the liver as free bile acids (FBAs) and then conjugated to either glycine or taurine *via* an amide bond. These conjugated bile acids (CBAs) are then released into the small intestine where they act primarily as emulsifiers of dietary lipid. Several genera of intestinal bacteria produce conjugated bile acid hydrolases (CBAHs) which cleave the amino acid moieties from CBAs. The resulting FBAs are less efficiently recycled by the enterohepatic circulation (1,2) and may be converted by further microbial action into potentially toxic bile acids (3,4). Because of the role CBAH plays in the metabolism of native and therapeutic bile acids in the body (5–8), our laboratory is pursuing biochemical studies of the CBAH reaction.

The CBAHs are a heterogenous group of enzymes having varied requirements for their CBA substrates. CBAHs from various bacterial sources have been shown to differ in their preferences for the amino acid moiety as well as for the hydroxyl group orientations on the steroid nucleus (9–13). The substrate specificity of the CBAH from *Clostridium perfringens* has been investigated by Batta *et al.* (14) and by Huijghebaert and Hofmann (15). The latter studies demonstrated that, although the rates and extent of hydrolysis varied according to the length of the side chain, the presence or absence of a negative charge, and steric hindrance around the amide bond, most substrates tested were hydrolyzed to some degree. Maeda *et al.* (16) used a conjugate of ursodeoxycholic acid and para-aminobenzoic acid to detect bacterial growth in the intestine. Initial experiments demonstrated that all individual bacterial strains tested which were capable of deconjugating glycocholic acid could also hydrolyze this synthetic conjugate. A notable exception to this general nonspecificity is the observation that amino acid conjugates of bile acids with longer side chains, such as those found in lower vertebrates and in humans with metabolic disorders, were not hydrolyzed by commercially available *C. perfringens* CBAH (17). For studies requiring the characterization of these longer side-chain bile acids, many of which are destroyed by alkaline hydrolysis, it would be advantageous to identify and make available CBAHs capable of hydrolyzing these conjugates.

One of the difficulties in performing screening and enzymatic studies with CBAH is that the current assay methods are time-consuming, all relying on end point (discontinuous) type assays. These assays are primarily based on the measurement of released free amino groups, most commonly by reaction of a deproteinated aliquot of the enzymatic reaction mix with either ninhydrin (12) or trinitrobenzenesulfonic acid (TNBS) (9). One potential problem with these amine reactive agents is the nonspecific nature of the reaction, prohibiting or complicating measurements in buffers containing amines and, in the case of TNBS, sulfhydryl groups. Yet another method uses radiolabelled substrate to detect activity (18). In addition to also being a discontinuous assay, this method is often impractical because of the limited number of radiolabelled CBAs available.

The CBAHs from *Lactobacillus plantarum* (19) and *C. perfringens* (20) have recently been cloned and sequenced and are homologous to a *Bacillus sphaericus* penicillin-V amidase (PVA) (21). The PVAs are industrially important enzymes, which, like the CBAHs, catalyze the hydrolysis of the amide bond of its substrate, in this case penicillin-V. In an ef-

*To whom correspondence should be addressed at Department of Microbiology and Immunology, School of Medicine, East Carolina University, Moye Boulevard, Greenville, NC 27858-4354.

Abbreviations: ANBA, 5-amino-2-nitro-benzoic acid; CA, cholic acid; CBA, conjugated bile acid; CBAH, conjugated bile acid hydrolase; FBA, free bile acid; PVA, penicillin-V amidase; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; UV, ultraviolet.

Copyright © 1995 by AOCS Press

fort to facilitate the detection and measurement of the PVA reaction, Kerr (22) recently reported the successful use of a conjugate of phenoxyacetic acid and 5-amino-2-nitro-benzoic acid (ANBA) in a spectrophotometric assay for PVA activity. This suggested to us that a substrate consisting of a conjugate of ANBA with a bile acid might similarly be used to measure the CBAH reaction. This report describes the synthesis of a conjugate of cholic acid (CA) and ANBA and a demonstration of its use in a continuous spectrophotometric assay to monitor CBAH activity.

EXPERIMENTAL PROCEDURES

Chemicals and enzymes. Clostridium perfringens CBAH (cholylglycine hydrolase), carboxypeptidase A, carboxypeptidase B, pancreatin, proteinase K, pronase E, and CA were purchased from Sigma Chemical Company (St. Louis, MO). Trypsin (TPCK-treated) was from Worthington Biochemical Corp. (Freehold, NJ). CA was filtered through celite/charcoal and recrystallized from ethanol–water until pure as judged by thin-layer chromatography (TLC). ANBA was purchased from TCI America (Portland, OR) and was recrystallized once from water before use. Ethylchloroformate and tri-*n*-butyl amine were purchased from Aldrich Chemical Company (Milwaukee, WI). Bulk chemicals and organic solvents were either purchased from Aldrich or from Fisher Chemical Company (Raleigh, NC) and were laboratory-grade. Machery-Nagel 0.25 mm thickness, silica gel-G TLC plates with ultraviolet (UV) indicator, were purchased from Alltech Associates, Incorporated (Deerfield, IL).

Analytical. Infrared spectra were determined as potassium bromide disks on a Perkin-Elmer 1605 infrared spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Absorbances are given as reciprocal centimeters (cm^{-1}). Proton nuclear magnetic resonance spectra were determined on a Varian-Gemini 200 MHz NMR spectrophotometer (Varian Analytical Instruments, Fernando, CA). Chemical shifts are given as parts per million (ppm) relative to tetramethylsilane, which was used as an internal standard.

Synthesis of CA–ANBA. The CA conjugate of ANBA was synthesized using a modification of the procedure used by Maeda *et al.* (16) to synthesize the para-aminobenzoic acid conjugate of ursodeoxycholic acid.

In a 50-mL round bottom flask equipped with a thermometer and a magnetic stirring bar, 0.82 g (2 mmol) CA was dissolved in 6 mL dioxane containing 0.48 mL (2 mmol) tri-*n*-butylamine. This solution was chilled to 8°C, and 0.20 mL (2.1 mmol) ethylchloroformate was added. The mixture was stirred at the same temperature for 15 min, and 0.37 g (2 mmol) ANBA in 2.0 mL 1 M NaOH was added to the vigorously stirring solution. After 20 min, the reaction was stopped by adding 40 mL deionized water, and the solution was acidified with 2 M HCl. The mixture was transferred to a 500-mL separatory funnel and extracted three times with 60-mL portions of ethyl acetate. The organic phases were pooled, washed with deionized water until neutral, dried over sodium sulfate, and evaporated under reduced pressure to yield a deep amber residue. CA–ANBA was separated from unreacted CA and free ANBA by dry column chromatography, using a nylon tube packed with silica gel (63–200 μM; 55 cm × 2.54 cm; Alltech) and developed with chloroform/methanol/acetic acid (8:1:1, by vol). After visualization under UV light, the region containing CA–ANBA was cut out of the column, eluted from the silica gel with 100% methanol, and the methanol evaporated to leave 200 mg light-tan powder. This material was dissolved in a small volume of ethyl acetate and washed once with 0.1 N HCl and several times with water. The organic layer was then evaporated under reduced pressure. This material was then used for melting point determinations, TLC analyses and kinetic studies. The structure of CA–ANBA is shown in Figure 1. Yield 26%. m.p. = 161–164°C. Infrared, cm^{-1}: 3327 (OH), 1703 (C=O, *CO*OH), 1697 (C=O, *CO*NH), 1550/1517, (NO_2). Proton nuclear magnetic resonance spectra (d_6-DMSO): 10.56 (*s*, 1H, COO*H*), 7.95 (*m*, 3H, aromatic), 4.35 (*bs*, 1H, CHO*H*-12), 4.15 (*d*, 1H, CHO*H*-7), 3.82 (*s*, 1H, C*H*OH-12), 3.62 (*s*, 1H, C*H*OH-7), 3.35 (*m*, 1H, C*H*OH-3), 3.18 (*s*, 1H, N*H*), 0.99 (*d*, $J = 5.6$ Hz, 3H, Me-21), 0.81 (*s*, 3H, Me-19), 0.60 (*s*, 3H, Me-18).

The purity of the CA–ANBA preparation was determined *via* TLC using the same solvent system as for the dry column

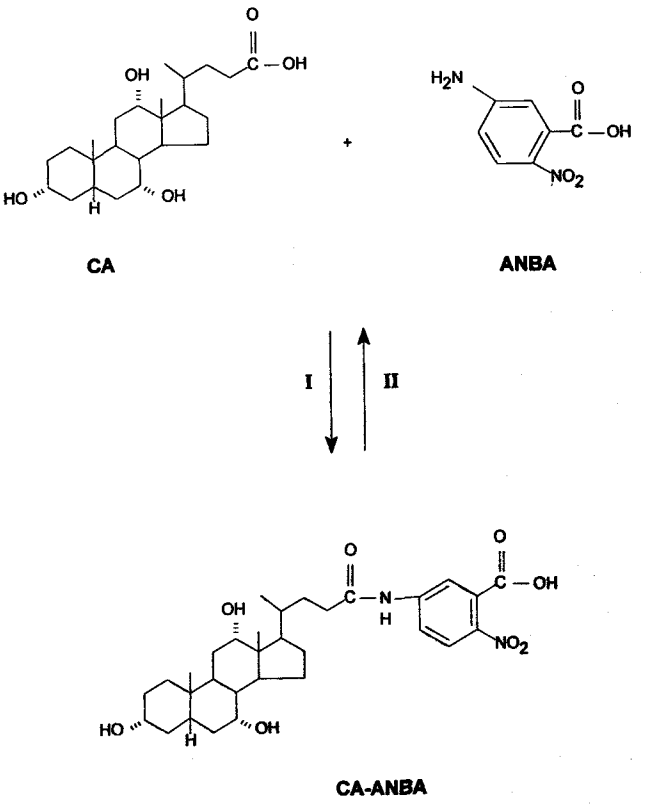


FIG. 1. Chemical synthesis and enzymatic hydrolysis of CA–ANBA. Pathway I, cholic acid (CA) and 5-amino-2-nitro-benzoic acid (ANBA) are conjugated *via* an amide bond in a reaction containing ethylchloroformate and tri-*n*-butlyamine in dioxane. Pathway II, hydrolysis of the product, CA–ANBA, by the enzyme, conjugated bile acid hydrolase.

chromatography. Free ANBA and its conjugate appeared as dark spots under short-wave UV light. CA and CA–ANBA were further visualized by spraying the plates with a 4% ethanolic solution of phosphomolybdic acid, then heating at 110°C for 10 min.

Spectrophotometric studies. All spectrophotometric assays were performed at 37°C on a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) equipped with a thermojacketted cuvette apparatus. Stock cholylglycine hydrolase was prepared according to the manufacturers instructions, to a concentration of 1000 units/mL (based on Sigma assay). Ninhydrin-based assays were performed according to the Sigma procedure. A unit of enzyme is defined by its rate of hydrolysis of glycocholic acid, since that amount hydrolyzes 1 μmol of glycocholic acid in 5 min at 37°C, pH 5.6. For the continuous assay, a stock solution of CA–ANBA (10 mM; theoretical molecular weight = 572.70) was prepared in 25 mM potassium phosphate buffer, pH 6.0. All reactions were carried out in 1 mL polystyrene cuvettes and contained, in a final volume of 1 mL: 5 μmol sodium phosphate, pH 5.6; 1 μmol EDTA; 1 μmol 2-mercaptoethanol; 0.5 μmol CA–ANBA; and enzyme. Reactions were initiated by the addition of enzyme to final concentrations ranging from 1.0 to 10.0 U per mL. The progress of these reactions was followed for 10 min at 410 nm. From these experiments, a concentration of 5 U/mL CBAH was chosen for subsequent kinetic studies. The initial velocity of hydrolysis of CA–ANBA at 10, 25, 50, 100, 200, 500, 750, and 1000 μM was measured. K_m values were calculated from these data.

The stability of stock solutions of CA–ANBA was assessed by incubating aliquots of the 10 mM CA–ANBA stock solution (prepared as described above and filter sterilized through a 0.2 μM filter) at 25°C under either light (fluorescent room lighting) or dark conditions and by monitoring the absorbance daily at 410 nm. The sensitivity of CA–ANBA to hydrolysis by other enzymes was assessed by monitoring the absorbance of solutions of CA–ANBA (0.5 mM) in 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0 containing either of the following enzymes at the indicated concentrations: carboxypeptidase A 15 U/mL), carboxypeptidase B (15 U/mL), pancreatin (1 mg/mL), trypsin (200 U/mL), pronase E (15 units/mL), and proteinase K (20 U/mL).

RESULTS AND DISCUSSION

Synthesis. The synthesis of CA–ANBA was accomplished by using a procedure initially used to synthesize glycine or taurine CBAs (16,23). The crude material obtained after acidification and extraction of the reaction mixture was found to have several unknown products of varying polarity. These were removed along with the unreacted ANBA and CA remaining in the mixture by dry column chromatography. The TLC R_f values for CA, free ANBA, and CA–ANBA were 0.33, 0.52, and 0.16, respectively. There was no contamination of the final product with either CA or free ANBA. Although we did not attempt to further optimize yields, it is possible that they could be improved by first protecting the bile acid hydroxy groups by formylation prior to reaction with ethylchloroformate.

Spectral properties. UV scans of CA–ANBA and ANBA showed maximum absorbances of 312 and 387 nm, respectively, with full resolution of the conjugate peak from the free ANBA peak at 410 nm. At this wavelength, the extinction coefficient of ANBA is 7180 M^{-1}. This extinction coefficient is of a magnitude sufficient to allow CBAH assay sensitivity similar to that of the ninhydrin- or TNBS-based assays. The trinitrophenyl derivatives of amino acids (amino acid–TNBS product) have extinction coefficients of approximately 20,000 M^{-1} (24), while the product of the ninhydrin reaction, diketohydrindylidenediketohydrindamine, has an extinction coefficient in its pure form of 22,000 M^{-1} (25). However, for ninhydrin-based assays, the color yield varies with reaction conditions and buffer composition. The typical standard curve for an amino acid such as glycine in the assay used by our laboratory correlates to an extinction coefficient of approximately 9,000 M^{-1} (26).

Enzyme kinetics. The rate of hydrolysis of 500 μM CA–ANBA by varying amounts of CBAH was followed for a period of 10 min (Fig. 2). The initial rate of hydrolysis of CA–ANBA to free ANBA increased linearly (r = 0.999) with increasing amounts of CBAH (Fig. 2, inset). When corrected for the time factor of the standard unit definition (1 unit hydrolyzes 1 μmol glycocholic acid in 5 min), the observed rate of absorbance change per unit of enzyme was 0.089 (5 min)$^{-1}$ U^{-1}, which corresponds to an ANBA formation rate of 12.5 nmol (5 min)$^{-1}$ U^{-1}. Thus, the relative velocity for hydrolysis of CA–ANBA was approximately 80-fold lower than that for hydrolysis of glycocholic acid.

Changing the substrate concentration with the amount of CBAH held constant provided rate measurements which

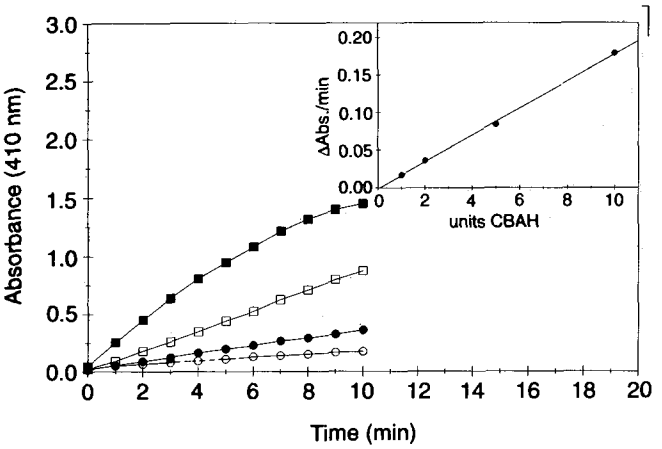


FIG. 2. Time course of hydrolysis of CA–ANBA. In a final reaction volume of 1 mL, CA–ANBA (500 μM) was incubated with increasing amounts of conjugated bile acid hydrolase (CBAH) as follows: 1 U/mL (○); 2 U/mL (●); 5 U/mL (□); and 10 U/mL (■). Inset: Regression analysis of the change in absorbance (Abs.) vs. units CBAH, calculated from the initial velocities of the hydrolysis of CA–ANBA. See Figure 1 for other abbreviations.

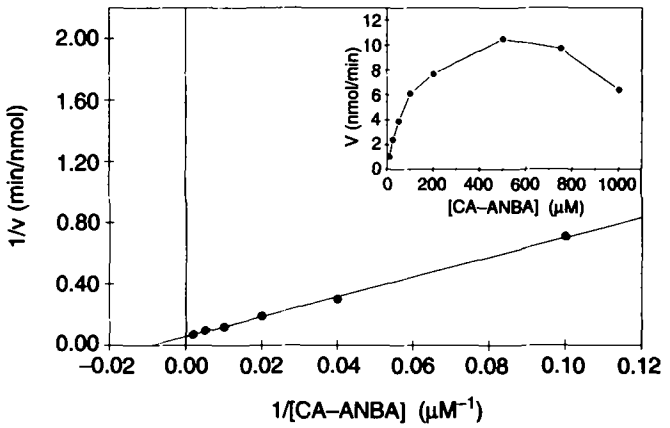


FIG. 3. Kinetic analysis of the hydrolysis of CA–ANBA by CBAH. Lineweaver-Burk plot using the data from 10–500 μM CA–ANBA. K_m = 120 μM. Inset: Michaelis-Menten plot of increasing velocity with constant CBAH concentration and 10, 25, 50, 100, 200, 500, 750, and 1000 μM CA–ANBA. Substrate inhibition occurs above 500 μM. See Figures 1 and 2 for abbreviations.

could be fitted to a classical Michaelis-Menten curve, with saturation of the enzyme and inhibition observed above 500 μM CA–ANBA (Fig. 3, inset). Double-reciprocal plots of the data (10–500 μM) yielded a straight line (r = 0.999) and a K_m value of 120 μM (Fig. 3). Previously reported K_m values of *C. perfringens* CBAHs for glycocholic acid range from 0.5 (9) to 8.0 mM (12). A visual comparison of reaction mixes at time zero and after the course of the reaction showed a distinct yellow color in the completed reaction, whereas the zero time point mixes appeared clear. Thus, this assay also can be used as a basic for a qualitative assessment of the presence of CBAH activity.

Stability to other enzymes and spontaneous hydrolysis. Neither carboxypeptidase A, carboxypeptidase B, crude pancreatin, trypsin, pronase E, nor proteinase K were active in hydrolyzing the amide bond of CA–ANBA. The chromogenic compound therefore appears to be similar to a normal CBA in its resistance to degradation by common proteolytic enzymes (15,27).

Spectrophotometric monitoring of CA–ANBA stock solutions over time indicated that they were relatively stable, showing less than 0.2% decomposition after two weeks under either light or dark storage conditions. Extrapolations from the data obtained over the two-week period indicated that the half life of buffered 10 mM solutions of CA–ANBA was greater than five years.

SUMMARY

The current methods of measuring CBAH activity are time-consuming, requiring a multiple-step procedure to detect the product. During the course of these experiments, it was shown that CA–ANBA was recognized with high affinity as a substrate for the *C. perfringens* CBAH. While the relative hydrolysis rate of CA–ANBA was 80-fold lower than that for glycocholic acid, the reaction produced an easily detectable product even at low concentrations of enzyme and substrate. CBAs are capable of deconjugating CBA substrates conjugated with a variety of moieties, even though with different degrees of efficiency. The results of Maeda *et al.* (16), which demonstrated that intestinal bacteria with CBAH activity could hydrolyze *p*-aminobenzoic acid conjugates of bile acids as well as natural CBA substrates, imply that the structurally related ANBA conjugates also may be generally susceptible to cleavage by these CBAHs. If this proves to be the case, conjugates of ANBA with CA, as well as other bile acids, will be useful substrates for studying the enzymology of CBAHs from a wide variety of bacterial sources. Since the free ANBA product absorbs in the visible region and thus can be monitored visually, the CA–ANBA conjugates reported here may also be useful as a rapid screening tool for the detection of microorganisms possessing CBA deconjugating activity. Kinetic and inhibitor studies can be performed by following CA–ANBA hydrolysis in the presence of natural bile acids or potential inhibitors. In this manner, relative affinities of various compounds for the CBAH can quickly be determined by virtue of their potential to compete with CA–ANBA for CBAH binding. Finally, the conjugation of bile acids or related molecules with ANBA may be generally applicable to the identification and characterization of hydrolases with altered substrate specificities.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Edson Rocha and Ho Young Kang for their helpful discussions and comments and Drs. Richard Marks and Art Rodriguez for valuable advice and critical review of the manuscript.

REFERENCES

1. Dietschy, J.M. (1974) Bile Acids: Their Absorption from the Gastrointestinal Tract and Role During Fat Absorption, *Verh. Dtsch. Ges. Inn. Med. 80*, 399–407.
2. Schiff, E.R., Small, N.C., and Dietschy, J.M. (1972) Characterization of the Kinetics of the Passive and Active Transport Mechanisms for Bile Acid Absorption in the Small Intestine and Colon of the Rat, *J. Clin. Inves. 43*, 467–476.
3. Bagheri, S.A., Bolt, M.G., Boyers, J.L., and Palmer, R.H. (1978) Stimulation of Thymidine Incorporation in Mouse Liver and Biliary Tract Epithelium by Lithocholate and Deoxycholate, *Gastroenterology 74*, 188–192.
4. Narisawa, T., Magadia, N.E., Weisburger, J.H., and Wynder, L.E. (1974) Promoting Effect of Bile Acids on Colon Carcinogenesis After Intrarectal Instillation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Rats, *J. Natl. Cancer Inst. 53*, 1093–1097.
5. Nakagawa, S., Makino, I., Ishizaki, T., and Dohi, I. (1977) Dissolution of Cholesterol Gallstones by Ursodeoxycholic Acid, *Lancet 2*, 367–369.
6. Roda, E., Bazzoli, F., Labate, A.M., Mazzella, G., Roda, A., Sama, C., Festi, D., Aldini, R., and Barbara, L. (1982) Ursodeoxycholic Acid vs. Chenodeoxycholic Acid as Cholesterol Gallstone-Dissolving Agents: A Comparative Randomized Study, *Hepatology 2*, 804–810.
7. Osuga, T., Tanaka, N., Matsuzaki, Y., and Aikawa, T. (1989) Effect of Ursodeoxycholic Acid in Chronic Hepatitis and Primary Biliary Cirrhosis, *Dig. Dis. Sci. 34*, 49S–51S.

8. Podda, M., Ghezzi, C., Battezzati, P.M., Bertolini, E., Crosignani, A., Petroni, M.L., and Zuin, M. (1989) Effect of Different Doses of Ursodeoxycholic Acid in Chronic Liver Disease, *Dig. Dis. Sci. 34*, 59S–65S.
9. Gopal-Srivastava, R., and Hylemon, P.B. (1988) Purification and Characterization of Bile Salt Hydrolase from *Clostridium perfringens*, *J. Lipid Res. 29*, 1079–1085.
10. Kobashi, K., Nishizawa, I., Yamada, T., and Hase, J. (1978) A New Hydrolase Specific for Taurine-Conjugates of Bile Acids, *J. Biochem. 84*, 495–497.
11. Lundeen, S.D., and Savage, D.C. (1990) Characterization and Purification of Bile Salt Hydrolase from *Lactobacillus* sp. strain 100-100, *J. Bacteriol. 172*, 4171–4178.
12. Nair, P.P., Gordon, M., and Reback, J. (1967) The Enzymatic Cleavage of the Carbon–Nitrogen Bond in 3α,7α,12α-trihydroxy-5β-cholan-24 oyl-glycine, *J. Biol. Chem. 42*, 7–11.
13. Stellwag, E.J., and Hylemon, P.B. (1976) Purification and Characterization of Bile Salt Hydrolase from *Bacteroides fragilis* subsp. *fragilis*, *Biochem. Biophys. Acta. 452*, 165–176.
14. Batta, A.K., Salen, G., and Shefer, S. (1984) Substrate Specificity of Cholylglycine Hydrolase for the Hydrolysis of Bile Acid Conjugates, *J. Biol. Chem. 259*, 15035–15039.
15. Huijghebaert, S.M., and Hofmann, A.F. (1986) Influence of the Amino Acid Moiety on Deconjugation of Bile Acid Amidates by Cholylglycine Hydrolase or Human Fecal Cultures, *J. Lipid Res. 27*, 742–752.
16. Maeda, Y., Takahashi, M., Tashiro, H., and Akazawa, F. (1989) The Rapid Evaluation of Intestinal Bacterial Growth Using a Conjugate of Ursodeoxycholic Acid with *para*-Aminobenzoic Acid, *J. Pharmacobio-Dyn. 12*, 272–280.
17. Batta, A.K., Salen, G., Cheng, F.W., and Shefer, S. (1979) Cleavage of the Taurine Conjugate of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic Acid by Rat Fecal Bacteria, *J. Biol. Chem. 254*, 11907–11909.
18. Feighner, S.D., and Dashkevicz, M.P. (1988) Effect of Dietary Carbohydrates on Bacterial Cholyltaurine Hydrolase in Poultry Intestinal Homogenates, *Appl. Environ. Microbiol. 54*, 337–342.
19. Christiaens, H., Leer, R.J., Pouwels, P.H., and Verstraete, W. (1992) Cloning and Expression of a Conjugated Bile Acid Hydrolase Gene from *Lactobacillus plantarum* by Using a Direct Plate Assay, *Appl. Environ. Microbiol. 58*, 3792–3798.
20. Coleman, J.P., and Hudson, L.L. (1995) Cloning and Characterization of a Conjugated Bile Acid Hydrolase Gene from *Clostridium perfringens*, *Appl. Environ. Microbiol.* 61:2514–2520.
21. Olsson, A., and Uhlén, M. (1986) Sequencing and Heterologous Expression of the Gene Encoding Penicillin V Amidase from *Bacillus sphaericus*, *Gene 45*, 175–181.
22. Kerr, D.E. (1993) A Colorimetric Assay for Penicillin-V Amidase, *Anal. Biochem. 209*, 332–334.
23. Bergström, S., and Norman, A. (1953) Synthesis of Conjugated Bile Acids, *Acta Chem. Scand. 7*, 1126–1127.
24. Fields, R. (1972) The Rapid Determination of Amino Groups with TNBS, *Methods Enzymol. 25*, 464–468.
25. Friedman, M., and Sigel, C.W. (1966) A Kinetic Study of the Ninhydrin Reaction, *Biochemistry 5*, 478–485.
26. Lee, Y.P., and Takahashi, T. (1966) An Improved Colorimetric Determination of Amino Acids with the Use of Ninhydrin, *Anal. Biochem. 14*, 71–77.
27. Huijghebaert, S.M., and Hofmann, A.F. (1986) Pancreatic Carboxypeptidase Hydrolysis of Bile Acid-Amino Acid Conjugates: Selective Resistance of Glycine and Taurine Amidates, *Gastroenterology 90*, 306–315.

[Received April 17, 1995, and in final revised form July 14, 1995; Revision accepted July 24, 1995]

Use of Gas Chromatography/Isotope Ratio-Mass Spectrometry to Study Triglyceride Metabolism in Humans

C. Binnert, M. Laville*, C. Pachiaudi, V. Rigalleau and M. Beylot

INSERM U 197, Faculté de Médecine Alexis Carrel, 69372 Lyon Cedex 08, and Department of Endocrinologie-Diabétologie et Nutrition, Hopital E. Herriot, 69437 Lyon Cedex 03, France

ABSTRACT: The study of triglyceride (TG) metabolism using stable isotope tracers would be facilitated by being able to detect low ^{13}C enrichment. To meet this goal, we developed a gas chromatography/isotope ratio-mass spectrometry technique to measure the enrichment of palmitate in nonesterified fatty acids (NEFA) and TG as its methyl derivative. This method allows accurate and reproducible measurements of enrichment as low as 0.009 mole percent excess (MPE), in a range between 0–0.65 MPE. The usefulness of this method is shown by two studies of lipid metabolism in human beings. First, we studied the metabolic fate of an oral TG load labeled with [1,1,1-$^{13}C_3$]tripalmitin. Labeled palmitate appeared concurrently in plasma NEFA and TG, and four hours after the load, the labeling was higher in NEFA than in TG (MPE NEFA: 1.53 ± 0.31 vs. MPE TG: 0.78 ± 0.06, $P < 0.05$). In a second study, the hepatic reesterification of NEFA was estimated by measuring the appearance of infused [1-^{13}C]palmitate in circulating TG. The estimated contribution of plasma NEFA to circulating TG increased to a maximum of 22%. Thus, gas chromatography/isotope ratio-mass spectrometry appears to be a useful tool for future studies of lipid metabolism in humans.
Lipids 30, 869–873 (1995).

Stable isotope tracers are being increasingly used for studies of lipid metabolism in humans (1,2). Measurements of the isotopic enrichment (IE) of plasma lipids are generally performed using gas chromatography/mass spectrometry/selected ion monitoring techniques (GC/MS/SIM). However, relatively high IE levels (1 mol%) are required to achieve accurate and reproducible measurements with GC/MS/SIM (1). Moreover, the study of some specific aspects of lipid metabolism requires the detection of low IE, because of the occurrence of an important dilution of the label during the metabolic process. This is the case for the determination of the metabolic fate of orally ingested triglycerides (TG) or the contribution of hepatic reesterification of plasma nonesterified fatty acids (NEFA) to TG secretion. The metabolic fate of ingested lipids has been studied with the vitamin A fat-loading test (3). This method allows investigators to study the metabolism of exogenous TG-rich lipoproteins, but not to trace the fate of the exogenous fatty acids themselves. Some studies have used a fat load labeled with radioactive tracers (4,5), but studies with stable isotopes require the ability to detect low IE in the fatty acids of the various circulating lipids. The contribution of the hepatic reesterification of NEFA to TG secretion has been addressed in one study using ^{14}C labeled fatty acids (6). To perform such studies with ^{13}C labeled fatty acids infusion, low IE should be measured in TG. Thus, to carry out these investigations and to avoid the use of an excessive amount of tracers, there is a need for a method that allows an accurate determination of low IE.

The development of a gas chromatography/isotope ratio-mass spectrometer (GC/IR-MS) (7,8) provides an attractive alternative to GC/MS/SIM. This technique has been shown to allow accurate determination of low IE levels in various metabolites such as glucose (8), lactate (9), and urea (10). Recently, using U ^{13}C labeled tracers in one human subject, Goodman and Brenna (11) were able to follow the appearance of orally ingested stearic acid in various lipoproteins fractions. Metges *et al.* (12) compared the enrichment of TG after ingestion of [1-^{13}C] or [8-^{13}C] triolein, and Brossard *et al.* (13) studied the compartmental metabolism of orally administered ^{13}C labeled docosahexaenoic acid in rats.

We report here that GC/IR-MS allows the determination of very low ^{13}C IE of fatty acids, using [1,1,1-$^{13}C_3$]tripalmitin or [1-^{13}C]palmitate as tracers. To demonstrate the usefulness of this method for *in vivo* human studies, two applications have been developed: (i) the metabolic fate of a triglyceride oral load labeled with [1,1,1-$^{13}C_3$]tripalmitin was investigated by the appearance of labeled palmitate in circulating NEFA and TG; and (ii) the hepatic reesterification of intravenously infused [1-^{13}C] palmitate was estimated from the appearance of labeled palmitate in TG.

*To whom correspondence should be addressed at INSERM U 197, Faculté de médecine Alexis Carrel, Rue Guillaume Paradin, 69372 Lyon Cedex 08, France.

Abbreviations: AP, ^{13}C atom percent; APE, atom percent excess; BMI, body mass index; GC/IR-MS, gas chromatography/isotope ratio-mass spectrometry; GC/MS/SIM, gas chromatography/mass spectrometry/selected ion monitoring; IE, isotopic enrichment; MPE, mole percent excess; NEFA, nonesterified fatty acids; PDB, Pee Dee Belemnite; TG, triglycerdes; TLC, thin-layer chromatography; ‰, per thousandth.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Material. [1,1,1-$^{13}C_3$]tripalmitin (99 atom% ^{13}C) and [1-^{13}C]palmitic acid (99 atom%,^{13} C) were purchased from Eurisotop (Saint Aubin, France). Chemical and isotopic purity were confirmed by GC/MS analysis. Pure, nonlabeled palmitate was obtained from Sigma (St. Louis, MO). Thin-layer chromatography (TLC) was performed on 60 G silica gel plates (Merck, Darmstadt, Germany). All solvents were from Merck. Boron trifluoride methanol (Sigma) was used for fatty acid methyl ester preparations.

Preparation of tracers. The addition of [1,1,1-$^{13}C_3$] tripalmitin to the sunflower oil was realized at 60°C in order to solubilize the tripalmitin, and the mixture was carefully homogenized. An aliquot of the labeled oil was collected to verify its enrichment.

[1-^{13}C]Palmitate was bound to human serum albumin (Mérieux, Lyon, France) as previously described (1). All infusates were checked for pyrogen by Limulus test before administration, and were infused through a 0.22 µm Millipore filter (Millipore, Bedford, MA).

Subjects. The appearance of [1-^{13}C]palmitate in circulating NEFA and TG, after the ingestion of a TG load labeled with [1,1,1-$^{13}C_3$]tripalmitin, was evaluated in six healthy subjects (five women and one man), aged 21–29 yr (mean 24 yr) with a body mass index (BMI) ranging from 19 to 24 (mean 21) kg/m^2.

During [1-^{13}C]palmitate infusion, the reesterification of NEFA was determined in four other subjects: three men and a woman aged 22–30 yr, mean 26 yr with a BMI ranging from 20 to 22 (mean 21) kg/m^2.

All subjects gave their written consent to the study after being informed of its nature, purpose, and possible risks. The experimental protocols were approved by the local ethics committee.

Experimental protocols. All tests were performed in the post absorptive state.

Appearance of [1-^{13}C]palmitate in circulating NEFA and TG after the ingestion of a TG load labeled with [1,1,1-$^{13}C_3$]-tripalmitin. On the morning of the test, an in-dwelling, nonheparinized catheter was placed into one forearm vein for blood sampling. After 30 min of bed rest and the collection of three basal samples, 30 g of sunflower oil enriched with 300 mg of [1,1,1-$^{13}C_3$]tripalmitin were ingested during five minutes. Blood was collected in tubes maintained at 4°C at 10, 20, and 30 min, and then every 30 min until 480 min.

Reesterification of NEFA in circulating TG. On the morning of the test, in-dwelling catheters were placed into a vein of one forearm, for [1-^{13}C]palmitate infusion, and in a vein in the opposite, for blood sampling. To obtain arterialized blood samples, this last catheter was placed in a retrograde manner and kept at 60°C in a warming blanket. Albumin bound [1-^{13}C]palmitate was infused at the rate of 0.04 µmol/kg/min after a bolus of 0.4 µmol/kg. This infusion lasted for 150 min. Blood samples were collected at 120 and 150 min in tubes kept at 4°C.

All blood samples were immediately centrifuged after collection, and plasma was stored at −20°C until the determination of metabolites, insulin concentrations, and IE of palmitate in circulating NEFA and TG.

Analytical procedures. Plasma glucose (14), NEFA (15), and TG (16) concentrations were measured by enzymatic methods. Plasma insulin was measured by radioimmunoassay (17). Total lipids were extracted from plasma according to Folch *et al.* (18) using methanol/chloroform (1:2, vol/vol). NEFA (R_f 0.22) and TG (R_f 0.5) were separated by TLC on 60 G silica gel plates using hexane/diethyl ether/acetic acid (80:20:1 by vol) as mobile phase (19). NEFA and TG were scraped off the plate and then treated with heptane/methanol (1:1, vol/vol) and 14% boron trifluoride in methanol at 100°C for 30 min for fatty acid methyl ester preparation (20).

GC/IR-MS analysis. Analyses were performed using an isotope ratio mass spectrometer (SIRA 12; VG Isogas, Middlewitch, United Kingdom) interfaced with a gas chromatograph (5890A; Hewlett-Packard, Evry, France) equipped with a Ross injector (230°C) and a capillary column (OV-1701, 0.25 mm i.d. × 25 m; Chrompack, Bridgewater, NJ). Oven temperature was maintained at 180°C for 3 min and then raised at 20°C/min to 230°C, where it was maintained for 5 min. Helium was used as carrier gas (injector inlet pressure, 13 psi). The gas chromatograph effluent was diverted to a flame-ionization detector until the elution of methyl palmitate. It was then switched inside a catalytic furnace (0.5-mm) filled with CuO and maintained at 800°C. The effluent of the furnace containing CO_2 and H_2O, flowing in the continuous helium flux, was driven into a water trap maintained at −100°C before its ionization by electron impact in the source of the IR-MS. Description of the GC/IR-MS has been given previously (8). Ions at *m/z* 44, 45, and 46 were continuously recorded until the return of the 44 signal to baseline value. Before and after the CO_2 peak arising from methyl palmitate, a standard CO_2 of known enrichment, calibrated vs. the international standard Pee Dee Belemnite (PDB), was automatically introduced into the spectrometer.

Calculations. The $^{13}C/^{12}C$ ratio of the sample and of the reference were used to calculate the ∂ per 1000 value (∂‰ ^{13}C) of the sample using the following formula:

$$\partial^{13}C‰ = \frac{(^{13}C/^{12}C)\text{sample} - (^{13}C/^{12}C)\text{ reference}}{(^{13}C/^{12}C)\text{ reference}} \times 1000 \quad [1]$$

where the reference is the international standard PDB.

The measured ∂‰ ^{13}C was transformed to ^{13}C atom % (AP) using the following formula:

$$AP = \frac{R \times [(0.001\ \partial^{13}Cs‰) + 1]}{1 + [R \times ((0.001\ \partial^{13}Cs‰) + 1)]} \times 100 \quad [2]$$

where R is the $^{13}C/^{12}C$ ratio of the international standard PDB

(R = 0.0112372) and ∂‰ ^{13}Cs, the ∂‰ ^{13}C value of the sample. The calculated AP of the palmitate in the labeled oil and in the plasma NEFA and TG were transformed into atom % excess (APE) using the following formula:

$$APE = APs - APb \quad [3]$$

where APs is the AP of the sample and APb is the AP of the plasma palmitate in the basal state.

^{13}C Enrichment of palmitate can be calculated from the measured ∂‰ either using a standard curve or the APE formula. However, it has to be taken into account that ∂ ^{13}C values measured on derivatized compounds are different from those of the respective underivatized molecules, and that there is only one ^{13}C carbon atom per molecule. The APE measured (APE m) could be thus converted to mole % excess (MPE) according to Khalfallah *et al.* (9): MPE = 17 APE (17 = number of carbon atoms of the derivatized molecule).

During the TG load study, the proportion of palmitate from exogenous origin found in circulating TG was determined at each time point as:

$$(MPE\ pp/MPE\ ip) \times 100 \quad [4]$$

where MPE pp is the MPE of palmitate in circulating plasma TG and MPE ip is the MPE of palmitate in the ingested triglycerides.

During the infusion of [1-^{13}C]palmitate, the percentage enrichment in TG relative to NEFA was determined after the measurement of the appearance of [1-^{13}C]palmitate in TG as:

$$= (MPE\ TG/MPE\ NEFA) \times 100 \quad [5]$$

where MPE TG is the value of the MPE of palmitate in plasma TG and MPE NEFA is the value of MPE of palmitate in plasma NEFA.

Palmitate flux was determined using steady-state equation:

$$F = (I/MR) - I \quad [6]$$

where *I* is the palmitate infusion rate (μmol/kg/min) and MR the molar ratio between ^{13}C palmitate and natural palmitate in plasma NEFA (mean value between 120 and 150 min).

Results are expressed as mean ± SEM. Comparisons were realized using the paired *t*-test or the nonparametric U test of Mann-Whitney when necessary.

RESULTS

In vitro *validation.* Natural ^{13}C abundance was measured on pure, commercially available methyl palmitate and on palmitate purified from plasma samples. Analyses were performed on 100–300 ng of methyl palmitate that gave a signal of similar intensity to the CO_2 standard. The sensitivity of the method allows one to utilize blood samples of small volume, as the treatment of 500 μL of blood permits 30 injections or more.

∂^{13}C‰ values were −28.4 ± 0.1 for pure methyl palmitate (Sigma) and −25.4 ± 0.4 and −25.8 ± 0.3 (NS) for methyl palmitate in human plasma NEFA and TG, respectively (mean of 12 samples) ($P < 0.001$ for methyl palmitate in NEFA or TG vs. methyl palmitate from Sigma). Interassay variation of ∂ ^{13}C‰ values obtained on pure methyl palmitate was tested by three daily injections over two weeks, and was 0.7% (∂^{13}C‰ mean ± SD −28.62 ± 0.20). The intraassay variability tested on ten successive injections was 0.5% (∂ ^{13}C‰ mean ± SD −28.59 ± 0.14).

Figure 1 shows a representative standard curve of [1-^{13}C]-palmitate diluted in natural palmitate (Sigma) obtained by GC/IR-MS analysis. The standard curve was linear from 0 to 0.65 MPE of [1-^{13}C]palmitate (r = 0.999, $P < 0.00001$) (Fig. 2A), including the part with low enrichment values (Fig. 1) (r = 0.999, $P < 0.00001$). With this technique, differences of 0.5 ∂ ^{13}C‰ = 0.009 MPE of [1-^{13}C]palmitate could be observed ($P < 0.005$, mean of three injections). No carryover of the high ^{13}C enrichment into low MPE samples was observed.

A strong correlation between MPE calculated from the above formula and MPE st deduced from the standard curve: $MPE_{st} = 1.01\ MPE_{th} + 0.009$ (r = 0.9999, $P < 0.0001$) was observed. Calculation of MPE gives results similar to those obtained using the standard curve, and thus, results could be expressed in MPE and the daily standard curve analysis avoided.

In vivo *studies. Appearance of [1-^{13}C]palmitate in circulating TG and NEFA after ingestion of a [1,1,1-$^{13}C_3$]tripalmitin labeled oil.* Figure 2 shows the evolution of TG and NEFA concentrations and the appearance of [1-^{13}C]palmitate in plasma TG and NEFA after the ingestion of [1,1,1-$^{13}C_3$]-tripalmitin labeled oil. Plasma TG concentration did not increase during the study, but a trend to an increase of NEFA concentration was observed six hours after the labeled TG load (Fig. 2A). No significant increase in insulinemia was observed (data not shown). [1-^{13}C]Palmitate appeared in plasma

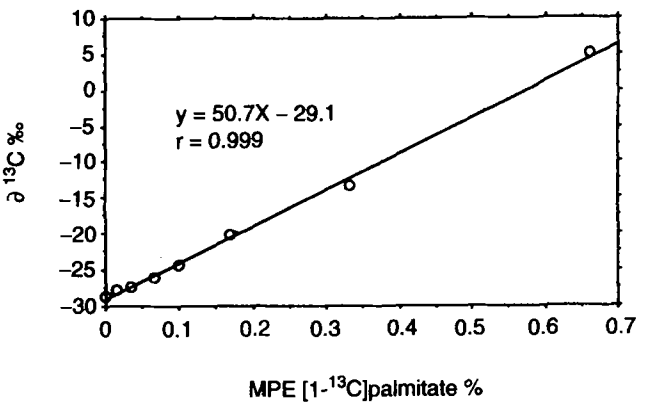


FIG. 1. A representative standard curve obtained by gas chromatography/isotope ratio-mass spectrometry. Palmitate was enriched with 0–0.65% mole percent excess (MPE) [1-^{13}C]palmitate. The measured ^{13}C enrichment is expressed as ∂ ^{13}C‰, referring to an international standard (Pee Dee Belemnite). Each point represents the mean of three analyses.

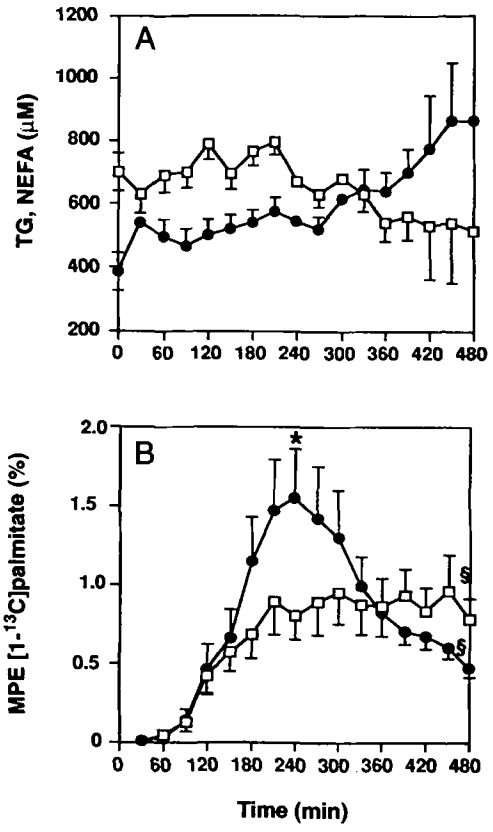


FIG. 2. Evolution of triglyceride (TG) and nonesterified fatty acid (NEFA) concentration (**A**) and of the appearance of [1-^{13}C] palmitate in plasma TG and NEFA (**B**) after the ingestion of a [1,1,1-$^{13}C_3$]tripalmitin labeled oil. ●, Indicates the NEFA, □ indicates the TG. Results are shown as the mean ± SEM. [1-^{13}C]Palmitate enrichment in TG were compared to [1-^{13}C]palmitate enrichment in NEFA, $^*P < 0.05$. [1-^{13}C]palmitate enrichment in TG and NEFA were compared to basal value. Other abbreviation as in Figure 1.

TG at 90 min, and the MPE of [1-^{13}C]palmitate in TG increased and reached a plateau after four hours. At the end of the study, MPE of [1-^{13}C]palmitate in TG remained significantly higher than baseline values ($P < 0.0001$) (Fig 2B). [1-^{13}C]Palmitate appeared in plasma NEFA initially following a similar kinetic to that in TG. Maximal values were obtained at 240 min, and at that time MPE of [1-^{13}C]palmitate was significantly higher in NEFA than in TG (MPE NEFA: 1.53 ± 0.3 vs. MPE TG: 0.78 ± 0.06, $P < 0.05$). Thereafter, it decreased to reach similar values to those of TG. Eight hours after the labeled load, the MPE of [1-^{13}C]palmitate in NEFA remained significantly higher than baseline values ($P < 0.0001$).

The value of each oil aliquot measured after derivatization was 12.6 ± 1.9 MPE. These values were not significantly different from the theoretical ones—the palmitate content of the sunflower oil was 7% (weight), thus the molar ratio of the load was 0.3/(30 × 7) = 14.3%. This demonstrates that a correct mixing of the labeled load was obtained.

The proportion of exogenous palmitate in circulating TG was maximal between four and five hours and was 0.4 ± 0.2, 3.1 ± 0.8, 5.4 ± 1.3, 7.4 ± 1.7, 7.0 ± 1.7, 6.7 ± 1.9, 5.5 ± 1.4, and 6.1 ± 1.4% at 1, 2, 3, 4, 5, 6, 7, and 8, respectively.

TABLE 1
Estimated Contribution from Nonesterified Fatty Acid (%) to Palmitate of Total Triglyceride

Time (min)	Subjects 1	2	3	4	Means ± SEM
120	17.3	16.6	3.2	18.7	13.9 ± 3.6
150	19.4	19.3	21.5	30.4	22.6 ± 2.6[a]

[a] $P < 0.001$.

Reesterification of NEFA during the infusion of [1-^{13}C]-palmitate. A constant ^{13}C palmitate enrichment of 1.6 ± 0.2 MPE was observed in NEFA after 120 min of [1-^{13}C]palmitate infusion. The NEFA turnover rate could be determined using steady-state equations and was 2.4 ± 0.2 μmol/kg/min.

In contrast, ^{13}C palmitate enrichment increased in plasma TG between 120 and 150 min from 0.23 ± 0.07 MPE to 0.37 ± 0.07 MPE ($P < 0.001$). Table 1 shows the individual values of the estimated contribution of palmitate from NEFA to palmitate of circulating total TG. This estimated reesterification increased from 13.9 ± 3.6 to 22.6 ± 2.6% between 120 and 150 min ($P < 0.001$).

DISCUSSION

An increase in the number of studies using stable isotopes has accompanied a growing interest in lipid metabolism. However, these investigations still are limited by the lack of accuracy in the measurement of low ^{13}C IE. We describe here, for the first time, a method using GC/IR-MS for the measurement of ^{13}C IE of palmitate. We demonstrate that this method is sensitive and reproducible as enrichments as low as 0.009 MPE of [1-^{13}C]palmitate could be detected and good inter- (0.7%) and intraassay variability (0.5%) are achieved. These characteristics are similar to those reported with the use of GC/IR-MS for measuring ^{13}C IE of glucose (8) or lactate (9). The validity of this method is further shown by the linearity of the results on a large range of enrichments, the concordance of measures of enrichments (12.6 MPE) in the labeled TG load with theoretical values, and by the similarity of palmitate turnover rate determined with this method, to those obtained in other studies using GC/MS (1). For many years we have used GC/MS measurements for the determination of palmitate turnover (1,21,22). The values obtained in healthy subjects were between 1.8 and 2.5 μmol/kg/min. Thus the value of 2.4 μmol/kg/min found in the present study using GC/IR-MS measurements was in agreement with the previous data.

In this study, we were able to estimate the metabolic fate of an oral TG load following the ingestion of [1,1,1-$^{13}C_3$]tripalmitin labeled oil. Significant labeling appeared concurrently both in TG and in NEFA. These findings agree with previous data from Havel and Frederickson (4), where ^{14}C palmitate was given to dogs, and significant radioactivity was

found in NEFA. They also agree with Roust and Jensen (5), who gave ^{14}C oleate to obese subjects and followed the appearance of exogenous oleate in NEFA. Our data are also in agreement with those of Griffiths *et al.* (21), demonstrating that the profile of plasma NEFA changed after a high-fat meal, consistent with the entry of meal-derived fatty acids into the plasma NEFA pool. These labeled NEFA could have come either from a direct release in portal circulation of fatty acids coming from the ingested TG or from an intravascular release of NEFA after the hydrolysis of TG-chylomicron by lipoprotein lipase. In contrast, it is widely accepted that the portal route is the main route for short-acid fatty acid chain (19), and that NEFA released by the lipoprotein lipase are taken up by tissues (20). To clarify the origin of the labeled NEFA, other studies using this GC/IR-MS method are required, including measurements of the labeling in TG chylomicrons.

In the second part of this study, we measured the appearance of infused [1-^{13}C]palmitate in circulating TG in order to estimate the hepatic reesterification of NEFA. The appearance of labeled palmitate in TG allowed us to estimate the conversion of NEFA into TG through the hepatic reesterification process. To our knowledge, few reports have addressed this question. In 1970, using [1-^{14}C]palmitate and hepatic venous sampling in healthy subjects, Havel and colleagues (6) have shown that similar specific activity was obtained in hepatic venous NEFA and arterial VLDL TG–fatty acids. These results differ from our data as they did not show more than 22% of TG coming from NEFA. However, the experimental conditions were different because, in our study, sampling was performed in peripheral arterialized blood and not in hepatic venous blood. Moreover, the contribution of NEFA to circulating TG increased from 120 to 150 min in our study; thus our experimental design might have been too short to reach the plateau. Further studies using GC/IR-MS are therefore required to better characterize the hepatic reesterification of NEFA in physiological and pathological states.

In conclusion, our report emphasizes the advantage of GC/IR-MS analysis for the study of palmitate metabolism in humans. Methods using [1-^{13}C]palmitate and [1-1-1-$^{13}C_3$]tripalmitin as tracers could be easily extended to other tracers:—[^{13}C]oleate, [^{13}C]linoleate—and should rapidly become a useful tool for lipid metabolism studies.

ACKNOWLEDGMENTS

We are indebted to M. Croset and Y. Khalfallah for their helpful advice and to M. Odéon for her excellent technical assistance. This work was supported by a grant from MRES n° 90 G 0750.

[Received February 6, 1995, and in final revised form June 23, 1995; Revision accepted July 10, 1995]

REFERENCES

1. Beylot, M., Martin, C., Laville, M., Riou, J.P., Cohen, R., and Mornex, R. (1991) *J. Clin. Endocrinol. Metab. 73*, 42–49.
2. Boden, G., Chen, X., Desantis, R.A., and Kendrick, Z. (1993) *Diabetes. 42*, 1588–1593.
3. O'Meara, N.M., Lewis, G.F., Cabana, V.G., Iverius, P.H., Getz, G.S., and Polonsky, K.S. (1992) *J. Clin. Endocrinol. Metab. 75*, 465–471.
4. Havel, R.J., and Frederickson, D.S. (1956) *J. Clin. Invest. 35*, 1025–1032.
5. Roust, L.R., and Jensen, M.D. (1993) *Diabetes 42*, 1567–1573.
6. Havel, R.J., Kane, J.P., Balasse, E.O., Segel, N., and Basso, L.V. (1970) *J. Clin. Invest. 49*, 2017–2035.
7. Matthews, D.E., and Hayes, J.M. (1978) *Anal. Chem. 50*, 1465–1473.
8. Tissot, S., Normand, S., Guilluy, R., Pachiaudi, C., Beylot, M., Laville, M., Cohen, R., Mornex, R., and Riou, J.P. (1990) *Diabetologia 33*, 449–456.
9. Khalfallah, Y., Normand, S., Tissot, S., Pachiaudi, C., Beylot, M., and Riou, J.P. (1993) *Biol. Mass Spectrom. 22*, 707–711.
10. Beylot, M., David, F., Khalfallah, Y., Normand, S., Parat, V., and Brunengraber, H. (1994) *Biol. Mass Spectrom. 23*, 510–513.
11. Goodman, K.J., and Brenna, J.T. (1992). *Anal. Chem. 64*, 1088–1095.
12. Metges, C.C., Kempe, K., and Wolfram, G. (1994) *Biol. Mass Spectrom. 23*, 295–301.
13. Brossard, N., Pachiaudi, C., Croset, M., Normand, S., Lecerf, J., Chirouze, V., Riou, J.P., Tayot, J.L., and Lagarde, M. (1994) *Anal. Biochem. 220*, 192–199.
14. Bergmeyer, H.U. (1974) *Methods of Enzymatic Analysis*, 3rd edn., p. 1228, Academic Press, New York.
15. Laville, M., Riou, J.P., Beylot, M., Perrot, L., Odeon, M., Berthezène, F. and Mornex, R. (1983) *Lyon Med. 249*, 67–70.
16. Bergmeyer, H.U. (1974) *Methods of Enzymatic Analysis*, 3rd edn., p. 1831, Academic Press, New York.
17. Hales, C.M., and Randle, J.P. (1963) *Biochem. J. 88*, 137–148.
18. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
19. Stein, J., and Smith G. (1982) *Introduction to Lipid Separation Methods*, pp. 1–13, Elsevier, North-Holland Publishing, Amsterdam.
20. Morrisson, W.R., and Smith, L.M. (1964) *J Lipid Res. 5*, 600–608.
21. Griffiths, A.J., Humphreys, S.M., Clark, M.L., Fielding, B.A., and Frayn, K.N. (1994) *Am. J. Clin. Nut. 59*, 53–59.
22. Beylot, M., Martin, C., Laville, M., Riou, J.P., Cohen, R., and Mornex, R. (1991) *J. Clin. Endocrinol. Metab. 73*, 42–49.

Microscale Synthesis of Phosphatidyl-[^{3}H]choline from 1,2-Diacylglycerol. Assessment of Isomerization by Reversed-Phase High-Performance Liquid Chromatography

Maurice Bugaut*, Joseph Gresti and Jerôme Gagniarre

Unité de Nutrition Cellulaire et Métabolique, Faculté des Sciences Mirande, Université de Bourgogne, 21004 Dijon, Cedex, France

ABSTRACT: The synthesis of *rac*-1-palmitoyl-2-oleoylglycero-3-phospho-[^{3}H]choline of high specific activity was carried out on a microscale by making 7 μmol of *rac*-1-palmitoyl-2-oleoylglycerol react first with an equimolar amount of $POCl_3$ and then of [^{3}H]choline. After purification by thin-layer chromatography and normal-phase high-performance liquid chromatography (HPLC), the yield of the synthesis of [^{3}H]phosphatidylcholine (120 μCi/μmol) was 22%. *rac*-1-Palmitoyl-2-oleoylglycerol was purified before use by reversed-phase HPLC under conditions which were nonisomerizing and allowed the separation of 1,2- and 1,3-isomers of diacylglycerol. Ethanol, but not benzene, was shown to cause isomerization of long-chain diacylglycerol and, therefore, was not used for drying the substrate before reaction. A rapid and complete separation of 1,2- and 1,3-isomers of long-chain phosphatidylcholine was obtained by reversed-phase HPLC using 20 mM choline chloride in methanol/acetonitrile/water (50:50:1, by vol) isocratically as the mobile phase. Under these conditions, analysis of the synthetized *rac*-1-palmitoyl-2-oleoylglycero-3-phospho-[^{3}H]choline showed a total absence of 1,3-isomer.

Lipids 30, 875–881 (1995).

Synthesis of phosphatidylcholine from diacylglycerol and choline was initially described by Baer and Kindler (1). A reactive intermediate, diacylglycerophosphooxychloride, is obtained by action of phosphorus oxychloride ($POCl_3$) on diacylglycerol in a mixture of anhydrous chloroform and pyridine. Addition of dry choline chloride leads to the formation of diacylglycerochlorophosphocholine, which is converted to phosphatidylcholine in the presence of water. The advantage of this method is the commercial availability of choline salt and of an array of various diacylglycerols in unlabeled and labeled form. The synthesis on a smaller scale (from 1–10 mg instead of 2.5 g diacylglycerol) was described by Myher and Kuksis (2). However, the large excess of choline chloride, used to compensate for the low solubility of dry powdered choline chloride in the solvent mixture, did not allow the synthesis of phosphatidyl-[^{3}H]choline with the high specific activity sometimes required for biochemical studies. The aim of the present study is to improve the microsynthesis of phosphatidylcholine with as little dilution of the starting [^{3}H]-choline chloride label as possible, while still keeping a satisfactory yield. Furthermore, isomerization of the substrate diacyl-*sn*-glycerol before its conversion into phosphatidylcholine was observed during drying, purification, and storage. The extent of this isomerization was investigated by analyzing diacyl-*sn*-glycerols by high-performance liquid chromatography (HPLC) under nonisomerizing conditions. An HPLC method also was developed for analysis and purification of the synthesized phosphatidylcholine. In this paper, the experimental conditions for the microsynthesis of phosphatidylcholine and resolution by reversed-phase HPLC of the 1,2- and 1,3-isomers of diacylglycerol and phosphatidylcholine are described. Also reported are results related to the extent of isomerization of diacyl-*sn*-glycerols under different experimental conditions.

MATERIALS AND METHODS

Samples and reagents. rac-1-Palmitoyl-2-oleoylglycerol and 1,3-dipalmitoylglycero-2-phosphocholine were purchased from Serdary Research Laboratories (London, Ontario, Canada). *sn*-1,2-Dioleoylglycerol, *sn*-1,2-dipalmitoylglycerol, dioleoylglycerol (*ca.* 89% 1,3-isomer), and *sn*-1,2-dipalmitoylglycero-3-phosphocholine were obtained from Sigma-Chimie (La Verpillière, France). [^{3}H-*Methyl*]choline chloride (85 mCi/μmol) was obtained from Commissariat à l'Energie Atomique (Saclay, France). Pyridine (0.01% water), $POCl_3$, and choline chloride were purchased from Merck (Darmstadt, Germany). Choline chloride was solubilized into ethanol and dried under vacuum before use. Anhydrous toluene, *(S)*-(+)-1-(1-naphthyl)ethyl isocyanate, and 4-pyrrolidinopyridine were obtained from Aldrich-Chimie (Strasbourg, France). Chloroform (SDS, Peypin, France) contained 0.4% ethanol and 0.015% water. When used for synthesis of

*To whom correspondence should be addressed at Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences Mirande, Université de Bourgogne, BP 138, 21004 Dijon, Cedex, France.

Abbreviations: HPLC, high-performance liquid chromatography; $POCl_3$, phosphorus oxychloride; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

phosphatidylcholine, chloroform was washed twice with distilled water and then allowed to dry over dry $CaCl_2$ for 12 h and then over dry Na_2SO_4 for an additional 12 h. Washed and dried chloroform and pyridine were distilled just before use while being shielded from moisture. All other solvents were of analytical reagent grade or HPLC analysis grade.

Purification of diacylglycerols. The sample of *rac*-1-palmitoyl-2-oleoylglycerol was purified by reversed-phase HPLC as described later (see Fig. 1B). The purity of the collected racemate was approximately 99% when checked by HPLC under the same conditions (see Fig. 1B, 2), and the fatty acid analysis showed 50.3% palmitate and 49.7% oleate. The proportions of the *sn*-1,2 and *sn*-2,3 enantiomers were 50.2 and 49.8%, respectively, when the racemate was analyzed by HPLC on a silica column in the form of chiral derivatives.

Pure 1,3-dioleoylglycerol was prepared by reversed-phase HPLC just before use from a sample of dioleoylglycerol containing 11% X-1,2-isomer and 89% 1,3-isomer. The acetonitrile/acetone solution collected was evaporated to complete dryness at 20°C for about 10 min. When the 1,3-dioleoylglycerol was analyzed by HPLC at this point, its purity was better than 99%. The samples of *sn*-1,2-dioleoylglycerol and *sn*-1,2-dipalmitoylglycerol contained less than 1% 1,3-isomer when analyzed by reversed-phase HPLC and, therefore, were not purified before experiments.

Microsynthesis of phosphatidylcholine. Choline chloride (8.2 μmol in 58 μL ethanol) was placed in a Reacti-Vial (Pierce Eurochemie, Rotterdam, Holland) reaction vial of 1.0 mL inner volume (tube A) and dried under nitrogen at 40°C. [^{3}H]Choline chloride (1 mCi in 1.0 mL of ethanol) was successively added and evaporated to dryness under nitrogen. Drying was improved by using first ethanol and, then, pyridine. A Reacti-Vial magnetic stirring bar (Pierce) and 100 μL pyridine were added, and the tube was closed with a Teflon-lined screw cap and connected *via* a dry $CaCl_2$ cartridge to a vacuum pump with a needle through the septum. Tube A was maintained under vacuum at 40°C for 12 h.

rac-1-Palmitoyl-2-oleoylglycerol was purified by reversed-phase HPLC just before use, and the solution collected corresponding to 4.3 mg (7.2 μmol) of diacylglycerol was transferred into another similar Reacti-Vial tube (tube B). The solvents were evaporated to dryness under nitrogen at 40°C, and drying was completed twice using benzene. A chilled mixture (100 μL) of chloroform/pyridine/$POCl_3$ (5.5:0.5:0.041, by vol), corresponding to 7.4 μmol $POCl_3$, was added with a syringe. Tube B was closed under nitrogen and allowed to stand for 1.5 h at 0°C and then for 1 h at room temperature. The solution was then quantitatively transferred into tube A through the septum with a needle-fitted syringe. The needle connected to the vacuum pump was withdrawn, and the two holes in the septum were closed with fast-drying glue. The mixture was stirred for 48 h at 30 ± 2°C. The reaction was stopped by adding 10 μL water and stirring for 0.5 h. The products were extracted according to Arvidson (3). The organic extract was passed through a small Na_2SO_4 column, dried under nitrogen, and stored in toluene/methanol (50:50, vol/vol) at –20°C.

The labeled phosphatidylcholine was first purified by thin-layer chromatography (TLC) using silica gel H (Merck) and chloroform/methanol/acetic acid/water (75:45:12:6, by vol) as the solvent mixture (4). The labeled phosphatidylcholine was extracted from the silica gel using chloroform/methanol/water/acetic acid (50:39:10:1, by vol). The suspension was allowed to stand for 2 h at room temperature before filtration through glass wool. The solution was then treated following Arvidson's procedure (3). The labeled phosphatidylcholine also was purified by normal-phase HPLC just before use to remove possible traces of lysophosphatidylcholine. About 1.4 mg of labeled phosphatidylcholine in 150 μL chloroform/methanol (1:1, vol/vol) was injected into a 40 cm × 4.5 mm i.d. column packed with 5 μm Lichrosorb Si60 particules (Merck). The phosphatidylcholine was isocratically eluted with acetonitrile/methanol/water (72:18:10, by vol) as the mobile phase at the rate of 2.5 mL/min, using a Varian model 5000 apparatus (Varian Associates, Sunnyvale, CA) equipped with an ultraviolet detector adjusted at 206 nm.

Nonradioactive choline chloride and *sn*-1,2-dipalmitoylglycerol were used during the microsynthesis attempts to preserve substrates. The reaction products were fractionated by TLC according to Myher and Kuksis (2). Phosphatidylcholine was extracted from the silica gel as described earlier, and chloroform/methanol (90:10, vol/vol) was used for extraction of diacylglycerol. The yield of synthesis was determined from quantitative fatty acid analyses of the diacylglycerol and phosphatidylcholine by gas chromatography.

Separation of 1,2- and 1,3-isomers of diacylglycerol and phosphatidylcholine by reversed-phase HPLC. 1,2- and 1,3-Diacylglycerols were separated on a 25 cm × 4 mm i.d. Lichrocart column packed with 4 μm Lichrospher 100RP-18 particles (Merck) and protected by a Lichrosorb RP-18 precolumn using acetonitrile/acetone (55:45, vol/vol) as the eluting mixture at a flow rate of 1.1 mL/min under isocratic conditions. The column was connected to a Waters model 6000A solvent delivery system (Waters, Milford, MA) equipped with a Waters model R401 differential refractometer. Diacylglycerol (200–500 μg) in acetone (approximately 30 μL) was injected at room temperature. Each isomer was identified by analysis of each collected HPLC peak by TLC on 5 wt% borate-impregnated silica gel G (Merck) with chloroform/acetone (95:5, vol/vol) as the developing solvent (5). Reference long-chain diacylglycerols (R_f 0.48 and 0.61 for 1,2- and 1,3-isomers, respectively) were used for comparison. Peak areas were measured during HPLC analyses using an Enica 21 (Delsi Instruments, Suresnes, France) integrator-calculator.

1,2- and 1,3-Phosphatidylcholines were separated by reversed-phase HPLC using 20 mM choline chloride in methanol/acetonitrile/water (50:5:1, by vol) as the mobile phase at a flow rate of 1.3 mL/min. Phosphatidylcholine was dissolved in methanol/acetonitrile (10:1, vol/vol) before injection. Other experimental conditions were similar to those used for separation of 1,2- and 1,3-diacylglycerols.

HPLC resolution of sn-*1,2- and* sn-*2,3-diacylglycerols as chiral derivatives.* Diacyl-*sn*-glycerols were derivatized in the form of (*S*)-(+)-1-(1-naphthyl)ethyl urethanes and were separated by HPLC on a silica column as described by Laakso and Christie (6). Diacylglycerol (300 µg in 300 µL toluene) was reacted with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (10 µL) in the presence of 4-pyrrolidinopyridine (approximately 10 µmol) overnight at 50°C. The products were dissolved in 2 mL hexane/diethyl ether (1:1, vol/vol) and washed twice with 2 mL 2N HCl and three times with water. After evaporating solvents under nitrogen, the derivatives were dissolved in diethyl ether and purified on a 25 mm × 10 mm i.d. Sep-pak silica cartridge (Millipore, St. Quentin en Yvelines, France). HPLC separation was carried out using a Waters Model 625 LC System together with a Waters Model 486 ultraviolet detector adjusted at 280 nm, and a Shimadzu Model C-R5A integrator. The diacylglycerol derivatives were eluted on a 40 cm × 4.5 mm i.d. column packed with 5 µm Lichrosorb Si60 particles (Merck) with hexane/2-propanol (99.5:0.5, vol/vol) at a flow rate of 1.2 mL/min. Chromatographic peaks were identified using derivatives of standard diacyl-*sn*-glycerols.

Isomerization of sn-*1,2- and 1,3-dioleoylglycerols.* Isomerization of *sn*-1,2- and 1,3-dioleoylglycerols was studied under several experimental conditions. Under standard conditions, *sn*-1,2-dioleoylglycerol (300 µg) stored in acetone (20 µg/µL) was dried under nitrogen and immediately redissolved in 0.5 mL of the solvent studied (see Table 2 later in paper). The solution was evaporated to dryness under a strong stream of nitrogen at 40°C for 4 min and was maintained in dry form under these conditions for different lengths of time (from 0 to 3 h). The dioleoylglycerol then was dissolved in acetone and analyzed by reversed-phase HPLC to determine the proportion of the newly-formed 1,3-isomer. In some experiments, before drying, *sn*-1,2-dioleoylglycerol remained in contact with the solvent at room temperature (20 ± 2°C) for 15 h. In other experiments, after drying, *sn*-1,2-dioleoylglycerol was stored under vacuum at 20°C for 15 h before being redissolved in acetone for HPLC analysis.

Gas chromatography. Lipids were converted into fatty acid methyl esters using BF_3 according to Slover and Lanza (7), and then were analyzed on a laboratory-made 30 m × 0.4 mm i.d. glass column coated with 20M Carbowax (Applied Science Laboratories, State College, PA), using methyl heptadecanoate as internal standard. The carrier gas was nitrogen at a flow rate of 2.5 mL/min, and the temperature was maintained at 195°C. The column was connected to a Packard model 417 chromatograph (Packard, Downers Grove, IL) equipped with a Ross injector and linked to an Enica-21 integrator-calculator (Delsi Instruments, Suresnes, France).

Radioactivity counting. Radioactivity in lipid samples was measured using Permafluor III scintillation liquid (Packard) and a Packard model Tri-Carb 300C scintillation counter. Unlabeled total lipids of liver were first added to the samples of phospholipid to solubilize phosphatidylcholine in the scintillation liquid. After evaporation of organic solvents, 0.2 mL methanol followed by 7 mL Permafluor III were added. The mixture was stirred and allowed to stand for 2 h before counting. Picofluor 15 scintillation liquid (Packard) was used for measuring radioactivity in aqueous samples.

RESULTS AND DISCUSSION

Synthesis of rac-*1-palmitoyl-2-oleoylglycero-3-phospho-[^{3}H]choline.* Under the experimental conditions described in the Materials and Methods section, 26% of the ^{3}H radioactivity was found in the organic phase when the synthesis products were extracted according to Arvidson (3). After resolution of the lipophylic products by TLC, 86.6% of the extracted tritium was in the phosphatidylcholine fraction, corresponding to a yield of 22.5% when calculated from the initial radioactivity. This means that 1.84 µmol [^{3}H]choline chloride was converted into the same amount of phosphatidyl-[^{3}H]choline, corresponding to a yield of 25.5% when calculated from the starting amount of *rac*-1-palmitoyl-2-oleoylglycerol (7.2 µmol). When phosphatidyl-[^{3}H]choline was thereafter purified by HPLC on a Lichrosorb Si60 column, 85% of the ^{3}H radioactivity was recovered in the phosphatidylcholine fraction. In the present study, the yield was therefore not much lower than what we obtained (30%) under other conditions when starting from 65 µmol *rac*-1-lauroyl-2-oleoylglycerol and 72 µmol [^{3}H]choline chloride (8).

Improvements in the microsynthesis of phosphatidylcholine. When the starting amount of diacylglycerol was as low as 2 mg and the synthesis was carried out based on the procedure of Baer and Kindler (1), and as modified by Myher and Kuksis (2), about a 1% yield was obtained. Water, even in trace amounts, eliminates the reactive chlorine groups of the intermediary product. In the initial procedure (1,2), choline chloride, which is a hygroscopic compound, was dissolved in ethanol and dried under nitrogen. When an additional drying step using pyridine was performed without changes in the other conditions, the synthesis yield increased to 22.7%. The yield reached 24.7% when tube A was connected to a vacuum pump through a device which evaporates solvents to complete dryness and re-introduces air dried on $CaCl_2$ into the vial just before synthesis.

The low solubility of choline chloride in a mixture of chloroform/pyridine also can affect the yield. The ratio of chloroform to pyridine was changed without modifying the $POCl_3$ concentration. When the ratio was 3:3, 5:1, 5.5:0.5, and 5.8:0.2 (vol/vol), the yield was equal to 1.5, 14.2, 25.1, and 13.1%, respectively, after 24 h, although choline chloride is known to be soluble in pyridine, but not in chloroform. Choline chloride did not seem to be completely dissolved in the mixture of chloroform/pyridine (5.5:0.5, vol/vol) after 24 h, but the reaction mixture became clear after 48 h. The latter time was therefore chosen to stop the reaction. When the reaction time was extended to 96 h, no change in the yield was observed, showing that no degradation of the synthesized phosphatidylcholine occurred in the vial during this period. The yield was improved when decreasing the total volume of the reaction medium from 0.65 mL to 100 µL, when always

adding the same amount of $POCl_3$ (7.4 μmol), but was independent of the inner volume (in the range of 1–10 mL) of the reaction vial.

*Resolution of diacyl-*sn*-glycerols by HPLC.* In this study, a good separation method for intact long-chain 1,2- and 1,3-diacylglycerols using reversed-phase HPLC was established. Figure 1A shows that *sn*-1,2- and 1,3-dioleoylglycerols were completely separated under isocratic conditions with acetonitrile/acetone (55:45, vol/vol) as the mobile phase. The 1,3-isomer was eluted before the *sn*-1,2-isomer, as also observed by Kondoh and Takano (9) on an octadecylsilyl column. These authors separated 1,2- and 1,3-distearoylglycerol by a method which requires ethanol/acetonitrile/water as the eluting mixture (potentially isomerizing, see previously), nonisocratic elution conditions, and a longer elution time (40 min). Separation of both isomers appears to be easier on a nonbonded silica column (10–15) than on a reversed-phase column (9,16). However, a mixture of diacylglycerols is not resolved by normal-phase HPLC according to the nature of the constituent acyl chains. Figure 1B shows that during the purification of *rac*-1-palmitoyl-2-oleoylglycerol on a reversed-phase column, the 1,3-isomer (10.3%) and 1,2-isomer (82.5%) were clearly separated from contaminating longer-chain diacylglycerols (7.2%) containing stearate (0.5% of total fatty acids). Figure 2 shows that a rapid and complete separation by normal silica-phase HPLC of the 1,3-, *sn*-1,2- and *sn*-2,3-isomers of dioleoyl-*sn*-glycerol as their urethane derivatives was achieved under conditions similar to those described by Laakso and Christie (6).

Isomerization of sn*-1,2-diacylglycerol in ethanol and other solvents assessed by HPLC.* Isomerization of diacyl-

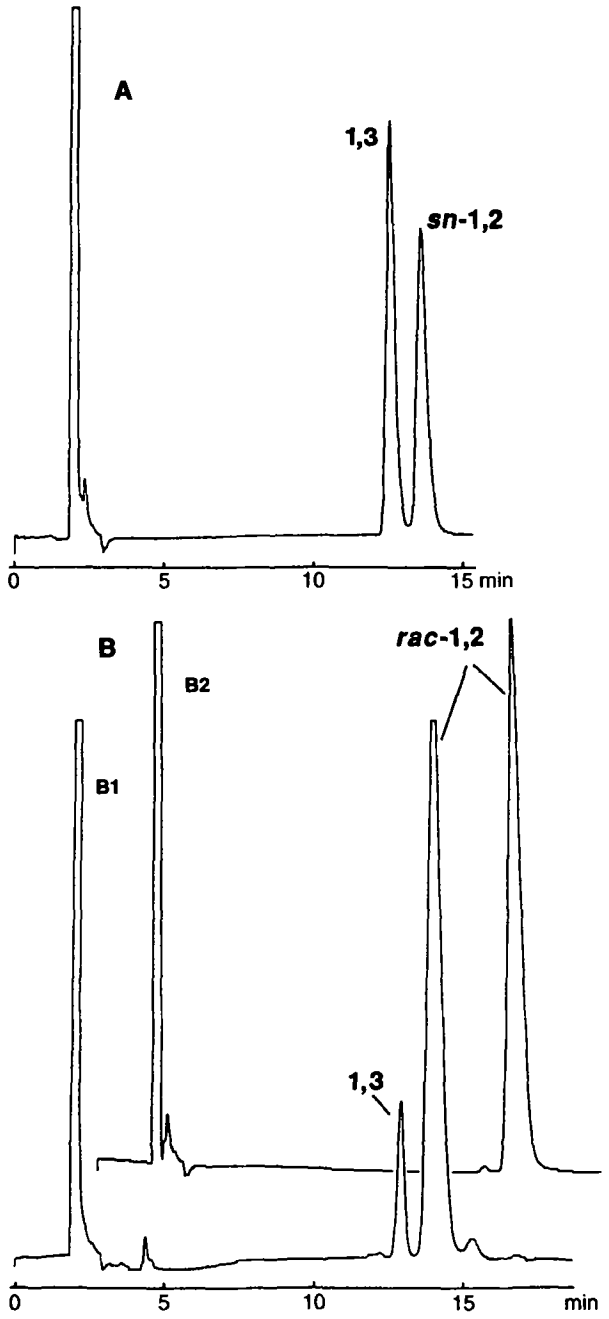


FIG. 1. Separation of the 1,2- and 1,3-isomers of synthetic diacylglycerols by reversed-phase high-performance liquid chromatography (HPLC) (conditions as in the text). A, Chromatogram of a mixture of pure *sn*-1,2- and 1,3-dioleoylglycerols (approximately 200 μg each). B, Chromatograms of *rac*-1-palmitoyl-2-oleoylglycerol before purification (1) and after purification (2) by reversed-phase HPLC.

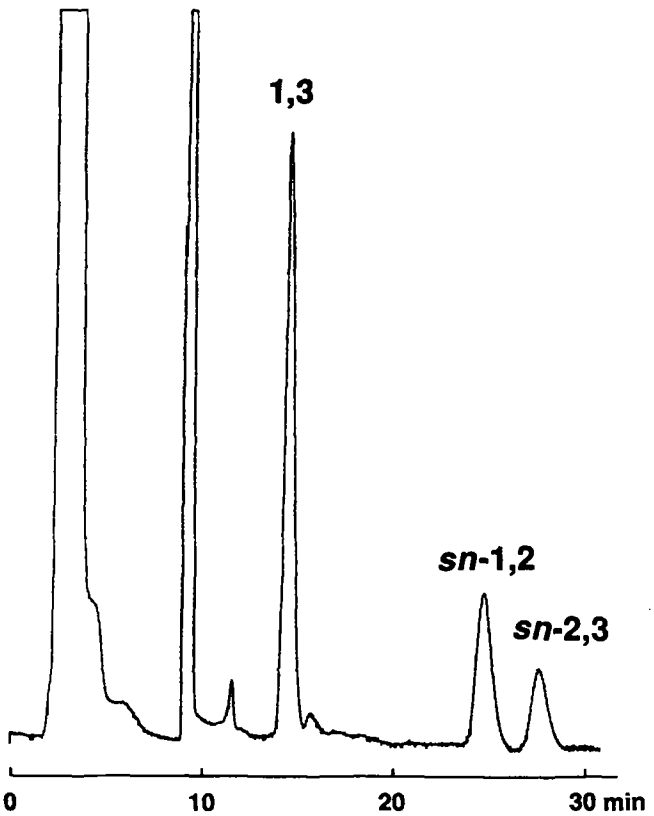


FIG. 2. High-performance liquid chromatography resolution of dioleoyl-*sn*-glycerols in the form of chiral urethane derivatives. Pure *sn*-1,2-dioleoylglycerol (300 μg) in 0.5 ml ethanol was dried under nitrogen at 40°C, maintained dry in this way for 3 h, and then redissolved in 300 μL toluene for derivatization with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate. Urethanes (100 μg in 50 μL 1,2-dichloroethane) were injected into a silica column (other conditions as in the text.)

glycerol is a reversible process (i.e., *sn*-1,2-, 1,3-, and *sn*-2,3-isomers), leading to an equilibrium mixture of 1,3- and X-1,2-isomers. Similar to that of monoacylglycerols (17,18), the isomerization of 1,2-diacylglycerol into its more thermodynamically stable 1,3-isomer is subject to general acid or base catalysis (19–22). This isomerization also is sensitive to certain reagents, including water (23,24), and may occur in crystalline state (25). In this study, effects of ethanol on isomerization of *sn*-1,2-dioleoylglycerol was examined first under the standard conditions described in the Materials and Methods section. The results obtained from 50 independent assays pointed out that the proportions of 1,3-isomer measured at times between 4 and 15 min were very scattered (from 1 to 67%). However, isomerization was complete within 20 min (*ca.* 65% 1,3-isomer) (Table 1). This result agrees with the fact that in long-chain diacylglycerols at equilibrium the ratio of the 1,2- to the 1,3-isomer is about 40:60 (20). In separate experiments, the dioleoyl-*sn*-glycerols were analyzed as chiral urethane derivatives under conditions known not to cause isomerization (6,26). Table 1 shows that the proportion (24%) of the *sn*-1,2-enantiomer remained higher than that (14%) of the newly-formed *sn*-2,3-enantiomer, even after a long period of drying (24 h).

Isomerization may start during evaporation of the dioleoylglycerol solution, or later when the dioleoylglycerol remains in dry form under nitrogen. Table 2 shows that the proportion (31.6%) of 1,3-isomer arising after a 15-min drying period was not significantly ($P > 0.10$) increased by storage under vacuum at room temperature overnight (46.9%). When *sn*-1,2-dioleoylglycerol was kept in ethanol at room temperature overnight before drying, no significant change in the results was observed (Table 2). Furthermore, 300 μg *sn*-1,2-dioleoylglycerol in 50 μL ethanol was maintained at 40°C for different lengths of time (from 0 to 3 h), and then was injected directly into the HPLC column. The proportion of 1,3-isomer (7.9 ± 14.1% as the average ± SD of 25 determinations) did not appear to depend on the length of contact of the diacylglycerol with ethanol. The results were not modified when *sn*-

TABLE 1
Effects of Ethanol on the Isomerization of *sn*-1,2-Dioleoylglycerol[a]

	Isomers		
Drying time[b]	*sn*-1,2	1,3	*sn*-2,3
0	96.7 ± 0.9	2.5 ± 0.6	0.8 ± 0.2
4 min	63.5 ± 13.8	31.7 ± 11.3	4.8 ± 2.5
20 min	28.3 ± 2.9	57.5 ± 3.4	14.2 ± 0.6
3 h	23.6 ± 2.1	62.0 ± 2.1	14.4 ± 0.6
24 h	23.4 ± 2.6	62.8 ± 1.9	13.8 ± 0.7

[a]Proportions of the dioleoyl-*sn*-glycerols determined by high-performance liquid chromatography on a silica column, in the form of chiral urethane derivatives. Average (mol%) ± SD of three independent observations.
[b]As described in the Materials and Methods section.

TABLE 2
Effects of Different Treatments on the Isomerization of *sn*-1,2-Dioleoylglycerol[a]

	Drying time			
	15 min	15 min	15 min	180 min
Solvents	Standard conditions[b]	Before drying, storage in solvents at 20°C for 15 h	After drying, storage under vacuum at 20°C for 15 h	Standard conditions[b]
Ethanol	31.6 ± 29.1 (0.0–65.4)	22.6 ± 25.1 (1.9–60.6)	46.9 ± 25.7 (4.7–68.3)	54.4 ± 7.7 (39.9–62.2)
Ethanol/water (100:20, vol/vol)		13.1 ± 4.8 (6.0–18.2)	32.8 ± 15.2 (21.7–55.0)	23.4 ± 14.7 (8.5–38.6)
Chloroform/methanol (90:10, vol/vol)	1.2[c]	2.4[c]	1.9[c]	14.8 ± 27.3 (1.2–64.4)
			3.4[c,d]	6.3 ± 1.7[d] (4.9–8.2)
Acetonitrile/acetone (55:45, vol/vol)	0.9 ± 0.8 (0.0–1.9)	0.8 ± 0.8 (0.0–1.7)	2.2 ± 1.9 (0.8–5.3)	4.4 ± 4.6 (1.1–11.6)
				3.2 ± 2.7[e] (1.3–7.8)
Acetonitrile/acetone/water (55:45:20, by vol)		3.6 ± 1.1[f] (2.2–4.7)	5.8 ± 3.2 (2.2–9.9)	9.2 ± 2.8 (5.7–13.3)
Benzene			0.9 ± 1.3 (0.0–2.9)	1.2 ± 0.6 (0.4–1.9)

[a]Proportions of the 1,3-isomer determined by reversed-phase high-performance liquid chromatography. Average (mol%) ± SD of five or more observations with the extreme values in parentheses.
[b]As described in the Materials and Methods section.
[c]One value.
[d]*sn*-1,2-Dioleoylglycerol was dissolved in 5 mL chloroform/methanol (90:10, vol/vol) before drying.
[e]The solution was dried using nitrogen saturated with water vapor.
[f]The tube was maintained under nitrogen until complete evaporation (>15 min).

1,2-dioleoylglycerol was purified beforehand by HPLC under nonisomerizing conditions to remove any trace of 1,3-isomer.

The suggestion that isomerization may arise at the moment when ethanol is nearly completely evaporated, inducing a momentary, high concentration of metals and other residues present in traces in ethanol, was investigated indirectly with different concentrations of *sn*-1,2-dioleoylglycerol ranging from 100 µg to 1.5 mg in 0.5 mL ethanol. After evaporation to complete dryness under nitrogen at 40°C for about 4 min, HPLC analyses were immediately performed. The proportion of the formed 1,3-isomer decreased significantly ($P < 0.05$) from 57.8 ± 8.6% (44.2–65.5% being the range of five determinations) to 30.5 ± 28.0% (1.2–66.5%) when the concentration of *sn*-1,2-dioleoylglycerol increased from 0.2 to 3.0 mg/mL. Moreover, experiments with ethanol purchased from Prolabo (Paris, France), Carlo Erba (Milan, Italy), and SDS led to similar results. Involvement of the solvents used for HPLC analyses, i.e., acetone and acetonitrile, in the isomerization described here can be ruled out (Table 2).

Results in Table 2 show that benzene is more reliable than ethanol in removing water traces in samples of *sn*-1,2-diacylglycerol. By contrast, some isomerization was observed when *sn*-1,2-dioleoylglycerol in acetonitrile/acetone was dried down, and then was maintained in dry form for 3–15 h. The purity of the nitrogen used for drying was questioned because traces of water could have been present. However, the isomerization rate did not change even when nitrogen saturated with water vapor (Table 2) or dried through a $CaCl_2$ cartridge was used. The potential role of water on the isomerization of *sn*-1,2-dioleoylglycerol was investigated further because water had been used as part of the mobile phase in reversed-phase HPLC (9), and has been demonstrated to provoke some isomerization (23,24). Table 2 shows that under all the experimental conditions studied, significant ($P < 0.05$) isomerization occurred when 20% water was added to the mixture of acetonitrile/acetone. By contrast, addition of 20% water to ethanol tended to significantly restrict isomerization when the solution of *sn*-1,2-dioleoylglycerol was stored overnight before drying ($P < 0.05$) or maintained dry under nitrogen for 3 h ($P < 0.001$), in comparison with samples of *sn*-1,2-dioleoylglycerol in ethanol alone (Table 2). Table 2 also indicates that precautions have to be taken to avoid isomerization when chloroform/methanol mixtures are used to extract diacylglycerol from silica gel after TLC and are then evaporated to dryness.

Isomerization of 1,3-dioleoylglycerol. When 1,3-dioleoylglycerol was purified by reversed-phase HPLC, the collected acetonitrile/acetone solution was evaporated cautiously under nitrogen at 20°C for 10 min, which caused no isomerization. However, evaporating the solution at 40°C for 15 min was sufficient to cause some isomerization (4.3 ± 2.5% X-1,2-isomer, 0.5–7.4% as the extreme values). The 1,3-isomer therefore appeared to be more sensitive to isomerization than the *sn*-1,2-isomer under similar conditions. When a solution of pure 1,3-dioleoylglycerol (300 µg) in 0.5 mL ethanol was dried under nitrogen at 40°C for 15 min, the proportion of formed X-1,2-isomer reached 26.0 ± 10.2% (8.5–36.6%). By contrast, Ikeda *et al.* (22) observed no isomerization of 1,3-dilauroylglycerol when the material was held at 80°C for 50 h in ethanol.

Resolution of 1,2- and 1,3-isomers of phosphatidylcholine by reversed-phase HPLC. sn-1,2-Dipalmitoylglycero-3-phosphocholine and 1,3-dipalmitoylglycero-2-phosphocholine were completely separated by reversed-phase HPLC under isocratic conditions at 17°C within a relatively short time (23 min) (Fig. 3A). The chromatogram in Figure 3B recorded during the analysis of labeled *rac*-1-palmitoyl-2-oleoylglyc-

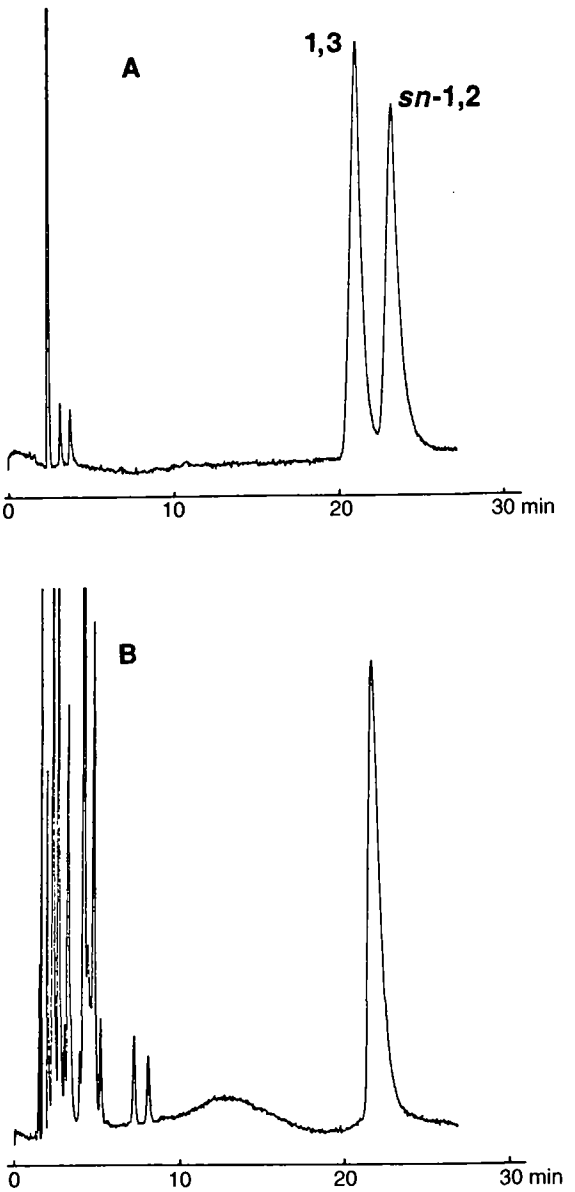


FIG. 3. Separation of synthetic 1,2- and 1,3-phosphatidylcholines by reversed-phase high-performance liquid chromatography (conditions as in the text). A, Chromatogram of a mixture of pure *sn*-1,2-dipalmitoylglycero-3-phosphocholine and 1,3-dipalmitoylglycero-2-phosphocholine (approximately 200 µg each). B, Chromatogram of the *rac*-1-palmitoyl-2-oleoylglycero-3-phospho-[^{3}H]choline synthesized as described in the Materials and Methods section.

ero-3-phospho-[^{3}H]choline displays a total absence of 1,3-isomer, which indicates that no isomerization occurred during the synthesis of the phosphatidylcholine. This result agrees with our previous finding (8) that less than 1% of *rac*-1,2-diacylglycero-3-phosphocholine, synthesized in a similar way, was resistant to phospholipases C and D.

In conclusion, the microscale synthesis of phosphatidylcholine from diacylglycerol and [^{3}H]choline chloride in the presence of $POCl_3$ has been improved to obtain a satisfactory yield and a high specific activity. The reversed-phase HPLC methods adapted here are useful and convenient tools for the control of isomeric purity of the lipid substrate (diacylglycerol) and product (phosphatidylcholine), as well as for their purification.

ACKNOWLEDGMENTS

The authors thank Nathalie Bancod and Courtney Chitwood for preparation of the manuscript.

REFERENCES

1. Baer, E., and Kindler, A. (1962) L-α-(dioleoyl)Lecithin. An Alternate Route to Its Synthesis, *Biochemistry 1*, 518–521.
2. Myher, J.J., and Kuksis, A. (1979) Stereospecific Analysis of Triacylglycerols *via* Racemic Phosphatidylcholines and Phospholipase C, *Can. J. Biochem. 57*, 117–124.
3. Arvidson, G.A.E. (1968) Structural and Metabolic Heterogeneity of Rat Liver Glycerophosphatides, *Eur. J. Biochem. 4*, 478–486.
4. Skispki, V.P., Peterson, R F., and Barclay, M. (1964) Quantitative Analysis of Phospholipids by Thin-Layer Chromatography, *Biochem. J. 90*, 374–378.
5. Thomas, III, A.E., Scharoun, J.E., and Ralston, H. (1965) Quantitative Estimation of Isomeric Monoglycerides by Thin-Layer Chromatography, *J. Am. Oil Chem. Soc. 42*, 789–792.
6. Laakso, P., and Christie, W.W. (1990) Chromatographic Resolution of Chiral Diacylglycerol Derivatives: Potential in the Stereospecific Analysis of Triacyl-*sn*-glycerols, *Lipids 25*, 349–353.
7. Slover, H.T., and Lanza, E. (1979) Quantitative Analysis of Food Fatty Acids by Capillary Gas Chromatography, *J. Am. Oil Chem. Soc. 56*, 933–943.
8. Bugaut, M., Kuksis, A., and Myher, J.J. (1985) Loss of Stereospecificity of Phospholipases C and D upon Introduction of a 2-Alkyl Group into *rac*-1,2-Diacylglycero-3-phosphocholine, *Biochim. Biophys. Acta 835*, 304–314.
9. Kondoh, Y., and Takano, S. (1987) Analysis of Acylglycerols by High-Performance Liquid Chromatography with Post-Column Derivatization. IV. Simultaneous Analysis of Mono-, Di- and Triacylglycerols, *J. Chromatogr. 393*, 427–432.
10. Sinsel, J.A., LaRue, B.M., and McGraw, L.D. (1975) High-Pressure Liquid Chromatographic Analysis of Glyceride-Based Lubricants, *Anal. Chem. 47*, 1987–1993.
11. Riisom, T., and Hoffmeyer, L. (1978) High-Performance Liquid Chromatography Analyses of Emulsifiers: I. Quantitative Determinations of Mono- and Diacylglycerols of Saturated Fatty Acids, *J. Am. Oil Chem. Soc. 55*, 649–652.
12. Perrin, J.L., Prévot, A., Stolyhwo, A., and Guiochon, G. (1984) Utilisation d'un détecteur à diffusion de lumière dans l'analyse des corps gras par CLHP, *Rev. Fr. Corps Gras 31*, 495–501.
13. Abe, K., and Kogure, K. (1986) Accurate Evaluation of 1,2-Diacylglycerol in Gerbil Forebrain Using HPLC and *in situ* Freezing Technique, *J. Neurochem. 47*, 577–582.
14. Foglia, T.A., Vail, P.D., and Takashi, I. (1987) High-Performance Liquid Chromatographic Analysis of 1-Alkyl-2-acyl- and 1-Alkyl-3-acyl-*sn*-Glycerols, *Lipids 22*, 362–365.
15. Hamilton, J.G., and Comai, K. (1988) Separation of Neutral Lipid, Free Fatty Acid and Phospholipid Classes by Normal Phase HPLC, *Lipids 23*, 1150–1153.
16. Parris, N.A. (1978) Non-Aqueous Reversed-Phase Chromatography of Glycerides Using Infrared Detection, *J. Chromatogr. 149*, 615–624.
17. Martin, J.B. (1953) The Equilibrium Between Symmetrical and Unsymmetrical Monoglycerides and Determination of Total Monoglycerides, *J. Am. Chem. Soc. 75*, 5483–5486.
18. Buchnea, D. (1967) Acyl Migration in Glycerides. I. A Bimolecular Resonant Ion Complex as Intermediate in Acyl Migration of Monoglycerides, *Chem. Phys. Lipids 1*, 113–127.
19. Mattson, F.H., and Volpenhein, R.A. (1962) Synthesis and Properties of Glycerides, *J. Lipid. Res. 3*, 281–296.
20. Serdarevich, B. (1967) Glyceride Isomerizations in Lipid Chemistry, *J. Am. Oil Chem. Soc. 44*, 381–393.
21. Banschbach, M.W., Geison, R.L., and O'Brien, J.F. (1974) Use of [1-^{14}C]Acetic Anhydride to Quantitate Diglycerides: A New Analytical Procedure, *Anal. Biochem. 59*, 617–627.
22. Ikeda, I., Gu, X.-P., Miyamoto, I., and Okahara, M. (1989) Preparation of 1,3-Diacylglycerols and 1-Alkyl-3-acylglycerols in the Presence of Quaternary Ammonium Salt, *J. Am. Oil Chem. Soc. 66*, 822–824.
23. Welsh, C.J., and Cabot, M.C. (1987) *sn*-1,2-Diacylglycerols and Phorbol Diesters: Uptake, Metabolism, and Subsequent Assimilation of the Diacylglycerol Metabolites into Complex Lipids of Cultured Cells, *J. Cell. Biochem. 35*, 231–245.
24. Robbins, S.J., and Nicholson, S.H. (1987) Long-Term Stability Studies on Stored Glycerol Monostearate (GMS). Effects of Relative Humidity, *J. Am. Oil Chem. Soc. 64*, 120–123.
25. Lutton, E.S. (1972) Lipid Structures, *J. Am. Oil Chem. Soc. 49*, 1–9.
26. Krüger, J. , Rabe, H. , Reichmann, G. , and Rüstow, B. (1984) Separation and Determination of Diacylglycerols as Their Naphthylurethanes by High-Performance Liquid Chromatography, *J. Chromatogr. 307*, 387–392.

[Received March 16, 1994, and in final revised form June 26, 1995; Revision accepted July 3, 1995]

ANNOUNCEMENTS

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) Temperature and Diet Effects on Trout, *J. Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) Temperature and Diet Effects on Trout, in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., Temperature and Diet Effects on Trout, *J. Biol. Chem.*, in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

CALENDAR

October 1995

21st World Congress and Exhibition of the International Society for Fat Research, Oct. 1–5, The Hague, The Netherlands. Information: 21st ISF Congress Secretariat, P.O. Box 3489, Champaign, IL 61826-3489 USA (Fax: 1-217-351-8091).

7th Asian Congress of Nutrition, Oct. 7–11, 1995, Beijing, China. Information: Mr. Ma Shi-liang, Chinese Academy of Preventive Medicine, 27 Nan Wei Road, Beijing 100050, China (Phone: 86-1-3022960; Fax: 86-1-3170892).

American College of Nutrition 36th Annual Meeting, Oct. 13–15, 1995, Omni Shoreham Hotel, Washington, D.C. Information: American College of Nutrition, c/o Hospital for Joint Disease, 301 E. 17th St., New York, NY 10003 (Fax: 212-777-1103).

(continued on next page)

Lipidforum Seminar: Lipids in Animal Nutrition, Oct. 25–26, 1995, Research Centre Foulum, Tjele (Viborg), Denmark. Information: G. Lambertsen, Lipidforum, Breistølen 1, N-5035 Bergen, Norway (Fax: 4755-32 57 24).

November 1995

XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

February 1996

Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

July 1996

European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

July–August 1997

Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Preparation and Characterization of Glucosylated Aminoglycerophospholipids

A. Ravandi, A. Kuksis*, L. Marai and J.J. Myher

Banting and Best Department of Medical Research and Department of Biochemistry, University of Toronto, Toronto, Ontario M5G 1L6, Canada

ABSTRACT: Natural aminophospholipids were isolated from egg yolk and from human red blood cells. Glucosylated ethanolamine and serine phosphatides were prepared by exposing synthetic and natural aminophospholipids to glucose for 3–18 h at pH 7.4. The glucosylation products were resolved from parent phospholipids by normal-phase high-performance liquid chromatography and were identified by on-line mass spectrometry with an electrospray interface. The soft ionization method allowed us to detect the glucosylation products as molecular ions of the Schiff bases. The Schiff bases could be stabilized by sodium cyanoborohydride reduction. The molecular species of the ethanolamine and serine phosphatides reacted in proportion to their molar concentration in the mixtures. The yields of the glucosylation products varied with time of reaction and the concentration of glucose in the medium. At 50 mM glucose and 0.6 mg/mL phosphatidylethanolamine, 20% of the aminophospholipid was glycated in 18 h at 37°C.
Lipids 30, 885–891 (1995).

Advanced glycosylation is believed to be a major pathway for the post-translational modification of tissue proteins, which begins with the nonenzymatic addition of sugars (such as glucose) to the primary amino groups of proteins (1,2). Although the nonenzymatic glycation of the primary amino groups of proteins has been extensively investigated, little attention has been given to phospholipid glycation reactions. The presence of reactive primary amino groups in phosphatidylethanolamine (PE) and phosphatidylserine (PS) has prompted the hypothesis (3) that glucose might react with lipids to initiate advanced glycosylation. However, glycated aminophospholipids have not been specifically isolated from any of the sources.

In this study we have prepared the glucosylation products of PE and PS, which are Schiff bases formed by nonenzymatic attachment of glucose to the amino moiety of the aminophospholipid, and have characterized them by high-performance liquid chromatography (HPLC) and on-line mass spectrometry (MS) with electrospray ionization (ESI).

*To whom correspondence should be addressed at the Banting and Best Department of Medical Research, University of Toronto, 112 College St., Toronto, Ontario M5G 1L6, Canada.

Abbreviations: ELSD, evaporative light-scattering detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; PE, phosphatidylethanolamine; PS, phosphatidylserine; TLC, thin-layer chromatography.

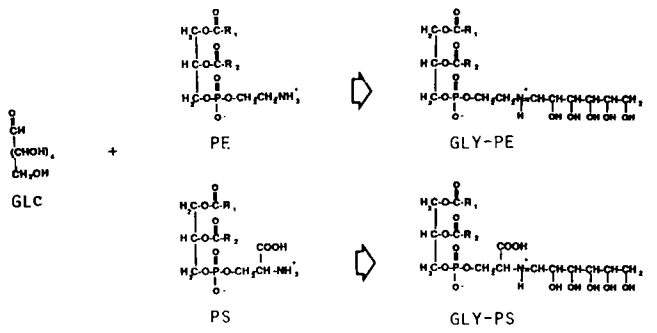
EXPERIMENTAL PROCEDURES

Materials. D-Glucose was obtained from BDH Chemicals (Toronto, Ontario). Synthetic *sn*-1,2-dipalmitoylglycero-3-phosphoethanolamine and bovine brain *sn*-1,2-dipalmitoylglycero-3-phosphoserine were purchased from Sigma Chemical Co. (St. Louis, MO). Egg yolk PE was isolated in the laboratory from chloroform/methanol (2:1, vol/vol) extracts of egg yolk by thin-layer chromatography (TLC) on Silica gel H (Merck, Darmstadt, Germany) using chloroform/methanol/acetic acid/water (75:35:12:6, by vol) as developing solvent (4). Human red blood cell phospholipids were extracted as described by Rose and Oaklander (5). Red cell PE and PS were isolated by TLC with chloroform/methanol/ammonia (30%) (65:35:7, by vol) as the solvent (6). The phospholipids were recovered from the silica gel by extraction with the developing solvent.

Glucosylation of aminophospholipids. Two mg of either PE or PS was dissolved in 1 mL methanol. Two mL of the glucosylation solution, which consisted of different concentrations of glucose (5–100 mM, in 0.1M phosphate buffer, pH 7.4) was added to this solution. The test tubes were intermittently sonicated for 30 min at room temperature to produce lipid emulsions. These emulsions were incubated on a mechanical shaker in the dark at 37°C for periods of 3–18 h. After incubation, the lipid-soluble material was separated from unreacted glucose by extraction with 2 mL chloroform/methanol (2:1, vol/vol). The tubes were centrifuged for 10 min and the organic phase was collected. The extraction was repeated two more times and the extracts were combined, blown down under nitrogen, and redissolved in chloroform/methanol (2:1, vol/vol) (Scheme 1). The glucosylations were performed several times at each glucose level and at each time interval.

Isolation of glucosylated aminophospholipids. The glucosylated and nonglucosylated lipids (1 mg) were resolved by TLC (20 × 20 cm plates, 250 micron thick layer of Silica gel H) using chloroform/methanol/ammonia (30%) (65:35:7, by vol). The resolved lipids were located by spraying the TLC

Copyright © 1995 by AOCS Press



SCHEME 1

plate with 2,7-dichlorofluorescein and viewing it under ultraviolet light. Both glucosylated and nonglucosylated lipids were recovered by scraping the gel from the fluorescent areas and extracting it with the developing solvent. After solvent evaporation, the residues were dissolved in chloroform or methanol.

$NaCNBH_4$ reduction. The glycated PE species were reduced by adding freshly prepared $NaCNBH_4$ in methanol (final concentration 70 mM). The sample was incubated at 4°C for 1 h. The product was washed three times with water to remove excess reagent.

Gas–liquid chromatography analysis of fatty acid methyl esters. Fatty acid methyl esters were prepared from aliquots of each sample resolved on the TLC plates by scraping the gel from the appropriate areas into 6% H_2SO_4 in methanol and heating at 80°C for 2 h. After the reaction, the methyl esters were extracted twice with hexane. The solvents were blown down under nitrogen and the samples redissolved in hexane. The fatty acids were analyzed on a polar capillary column (SP 2380, 15 m × 0.32 mm i.d., Supelco, Mississauga, Ontario, Canada) installed in a Hewlett-Packard (Palo Alto, CA) Model 5880 gas chromatograph equipped with a hydrogen flame-ionization detector (7). Hydrogen was the carrier gas at 3 psi.

HPLC of phospholipids. Normal-phase HPLC separations of phospholipids were performed on Spherisorb 3 micron columns (100 mm × 4.6 mm i.d.; Alltech Associates, Deerfield, IL) installed into a Hewlett-Packard Model 1050 Liquid Chromatograph connected to an evaporative light scattering detector (ELSD) (Model ELSD II; Varex, Rockville, MD). The column was eluted a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min (8). The flow rate was set at 1 mL/min.

Liquid chromatography (LC)/MS of phospholipids. Normal-phase LC/MS was performed by admitting the entire HPLC column effluent into a Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted ESI interface (HP 59987A). Negative ESI spectra were taken in the mass range 400–1100. The capillary exit voltage was set at 150 volts, with the electron multiplier at 1795 volts. Selected ion spectra were retrieved from the total ion spectra by computer. The masses given in the tables and figures are the actual masses of the ($[M - H]^-$) ions. The nominal masses are one unit lower.

The molecular species of the various glycerophospholipids were identified on the basis of the molecular mass provided by MS, the knowledge of the fatty acid composition of the aminophospholipid classes, and the relative HPLC retention time (more saturated species migrating ahead of the unsaturated species) of the phospholipids.

Figure 1 shows that on normal-phase TLC the glucosylated phospholipids migrate more slowly than the corresponding nonglucosylated derivatives, and that there is an effective resolution of the glucosylated aminophospholipids from other common phospholipids. The R_f values of PE and PS were 0.69 and 0.21, and 0.46 and 0.09 of glucosylated PE and glucosylated PS, respectively. Transmethylation of the TLC bands showed identical fatty acid composition for the corresponding precursor and product molecules.

Figure 2 shows the normal-phase HPLC separation of glucosylated PE relative to PE and other red blood cell phospholipids. The glucosylated PE is eluted later than nonglucosylated PE. Furthermore, the glucosylated PE is seen to overlap in part with phosphatidylcholine. Similarly, glucosylated PS was eluted later than nonglucosylated PS, both being eluted after the glucosylated PE (discussed later). The peaks were detected by ELSD, and their identity was confirmed by on-line MS.

Figure 3 shows that the yield of the glucosylated product depends on the concentration of the glucose in the incubation medium. Detectable glucosylation was seen at 5 mM, which corresponds to the glucose level in healthy subjects (9). The 50-mM glucose yielded 15–20% glucosylation in 18 h. In-

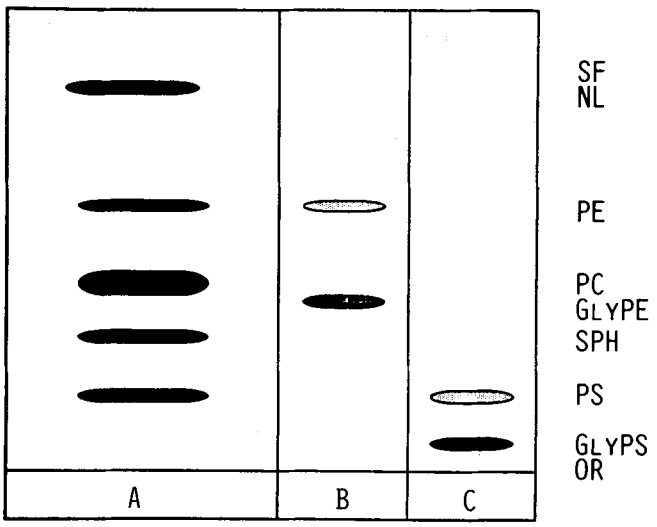


FIG. 1. Thin-layer chromatography of common phospholipids before and after glucosylation with 50 mM glucose. A, reference phospholipids; B, glucosylated phosphatidylethanolamine (PE); C, glucosylated phosphatidylserine (PS); SF, solvent front; NL, neutral lipid; PC, phosphatidylcholine; SPH, sphingomyelin; GLYPE, glucosylated PE; GLYPS, glucosylated PS; OR, origin. Solvent: chloroform/methanol/ammonia (30%) (65:35:7, by vol); detection: 2,7-dichlorofluorescein.

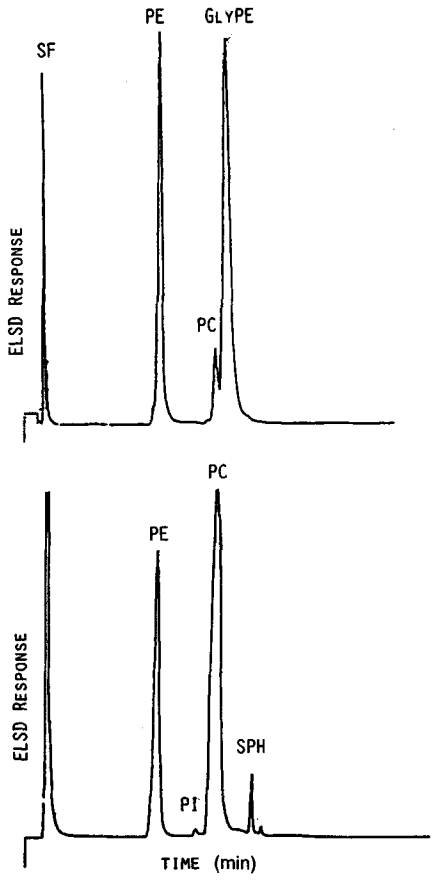


FIG. 2. Normal-phase high-performance liquid chromatography of glucosylated and nonglucosylated phospholipids. A, partially glucosylated egg yolk PE; B, nonglucosylated red blood cell phospholipids. Solvent: linear gradient of 100% Solvent A to 100% Solvent B in 14 min, then Solvent B for 10 min. Solvent A: chloroform/methanol/30% ammonium hydroxide (80:19.5:0.5, by vol); Solvent B, chloroform/methanol/water/30% ammonium hydroxide (60:34:5.5:0.5, by vol). Detection: evaporative light-scattering detection (ELSD). Abbreviations as in Figure 1; PI, phosphatidylinositol. PS and PC overlap in this solvent system.

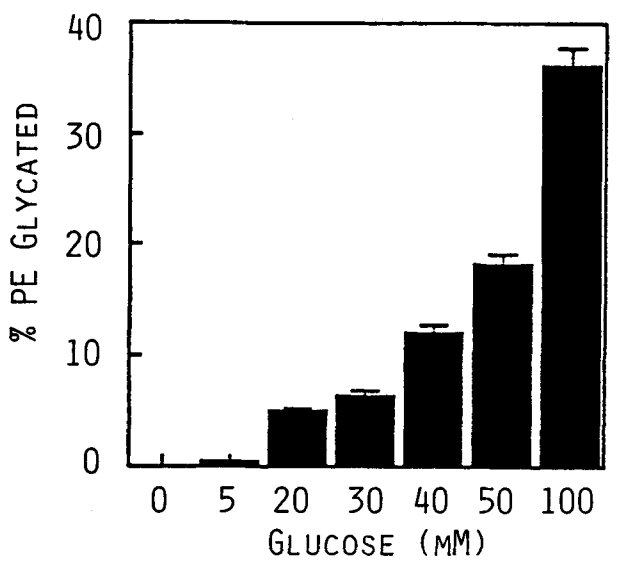


FIG. 3. Formation of glucosylated PE during an overnight (18 h) incubation (37°C, pH 7.4) with various concentrations of glucose. The glucosylated and nonglucosylated PE were quantitated by normal-phase high-performance liquid chromatography with ELSD. Abbreviations as in Figures 1 and 2.

creasing the glucose concentration to 100 μM doubled the yield of the Schiff base, and further increases in glucose led to additional glucosylation of the PE. The small SD values show that the glucosylation is reproducible. Similar results were obtained with PS.

Figure 4 shows the total negative ion current profile and full spectra as obtained by LC/ESI/MS for the dipalmitoyl species of PE after glucosylation and $NaCNBH_4$ reduction and the dipalmitoyl species of PS after glucosylation. In each instance, the symmetrical ion current profiles are accompanied by single $[M - H]^-$ ions of the correct mass. Following reduction, the glucosylated compounds were stable to acid and showed an increase in the *m/z* value of 2 amu, indicating a saturation of the original Schiff base structure. There was a slight increase in the retention time of the reduced, in comparison to the nonreduced, glucosylation products.

Figure 5 shows the total ion current profile obtained by normal-phase LC/MS for a mixture of nonglucosylated and glucosylated (18 h) egg yolk PE, along with the electrospray spectra averaged over the peaks of the two types of PE molecules. The egg yolk PE was incubated with 25 mM glucose. The major ions for the nonglucosylated PE are *m/z* 715 (16:0–18:2), *m/z* 717 (16:0–18:1), *m/z* 739 (16:0–20:4), *m/z* 743 (18:0–18:2 and 18:1–18:1), *m/z* 767 (18:0–20:4), and *m/z* 793 (18:0–22:5), corresponding to the major diacyl species in the original egg yolk PE (10). There are also minor ions corresponding to alkenylacyl species, e.g., *m/z* 775 (18:0–22:6), *m/z* 777 (18:0–22:5), and *m/z* 779 (18:0–22:4). The major molecular ions for the glycated PE are *m/z* 877 (16:0–18:2), *m/z* 879 (16:0–18:1), *m/z* 901 (16:0–20:4), *m/z* 905 (18:0–18:2 and 18:1–18:1), *m/z* 907 (18:0–18:1), and *m/z* 929 (18:0–20:4). It appears that the oligoenoic species of PE are glucosylated as readily as the polyunsaturated and the alkenylacyl species.

Figure 6 shows the normal-phase LC/MS profile of human red blood cell phospholipids following incubation with 50 mM glucose (18 h), along with the mass spectra averaged over the glucosylated and nonglucosylated PE and PS subclasses. The total ion current profile of nonglucosylated PS shows two peaks, because the more unsaturated species migrate slightly faster than the more saturated species. This difference is not apparent following glucosylation when they overlap. About 50% of each phospholipid class was glucosylated. The PE showed glucosylation of both the diacyl and the alkenylacyl species. The molecular species of the PE and PS were glucosylated roughly in proportion to their mole percentages in the phospholipid classes in the human red blood cell. The major component in both nonglucosylated PE (*m/z* 751) and in glucosylated PE (*m/z* 913) was the alkenylacyl (18:0–20:4) species. The major component in both nongluco-

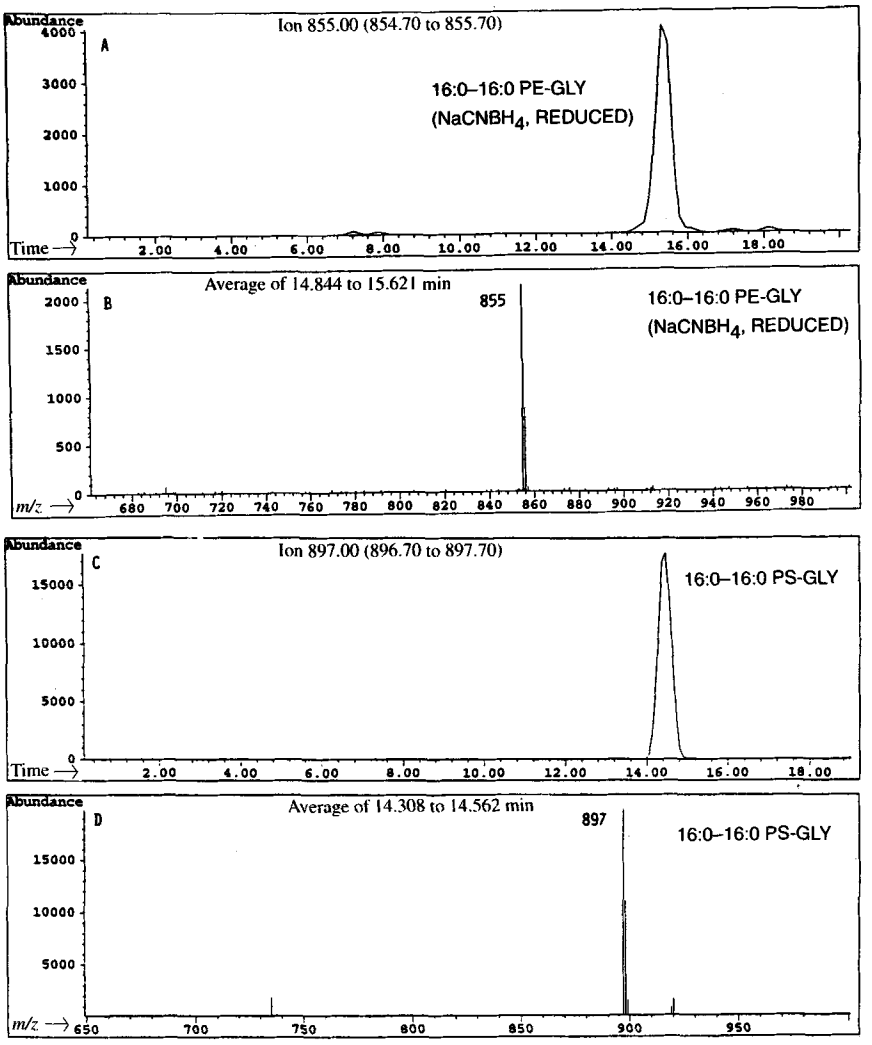


FIG. 4. Normal-phase liquid chromatography/mass spectrometry (MS) with electrospray ionization of glucosylated and $NaCNBH_4$-reduced dipalmitoyl aminophospholipids. Selected ion plot (A) and full spectrum (B) of glucosylated and reduced PE; selected ion plot (C) and full spectrum (D) of glucosylated PS. High-performance liquid chromatography and MS conditions are given in Figure 2 and in the Experimental Procedures section. Abbreviations as in Figure 1.

sylated PS (*m/z* 811) and in glucosylated PS (*m/z* 973) was the diacyl (18:0–20:4) species. Table 1 lists the $[M - H]^-$ values, identities, and the relative proportions of the major molecular species in the glucosylated and nonglucosylated portions of the aminophospholipids. The proportions of the major molecular species within the glucosylated and nonglucosylated forms of each aminophospholipid class were calculated from the $[M - H]^-$ ion (corrected for M + 1 and M + 2, where necessary) intensity and are given as percent of total. These proportions agreed closely with the known composition of the major species of egg yolk and red blood cell PE and PS (11). The proportions of glucosylated and nonglucosylated forms of each aminophospholipid were determined by normal-phase HPLC with ELSD. The ESI intensity differed significantly among the different phospholipid classes and between the glucosylated and nonglucosylated PE and PS, and required appropriate correction during quantitation in either positive or negative ion mode.

DISCUSSION

Although glucose is the least reactive of the common monosaccharides in Schiff base formation (12), it has been suggested that it might react with aminophospholipids and play a role in production of age pigments. The products of the reaction, however, have not been isolated. Previous attempts had not proven successful in the isolation of glycated PE from phospholipid-containing membranes (9). The earlier failure to isolate glycated PE may have been due to the easy dissociation of the Schiff base in the presence of mild acid, which is frequently employed in lipid extraction and TLC of phospholipids. We have not determined the exact time course of acid hydrolysis of glucosylated aminophospholipids. However, we have repeatedly observed the decomposition of the glucosylation products during extraction and chromatography (HPLC and TLC) with solvents containing small amounts of acetic, trifluoroacetic, or phosphoric acid. In contrast, these solvent

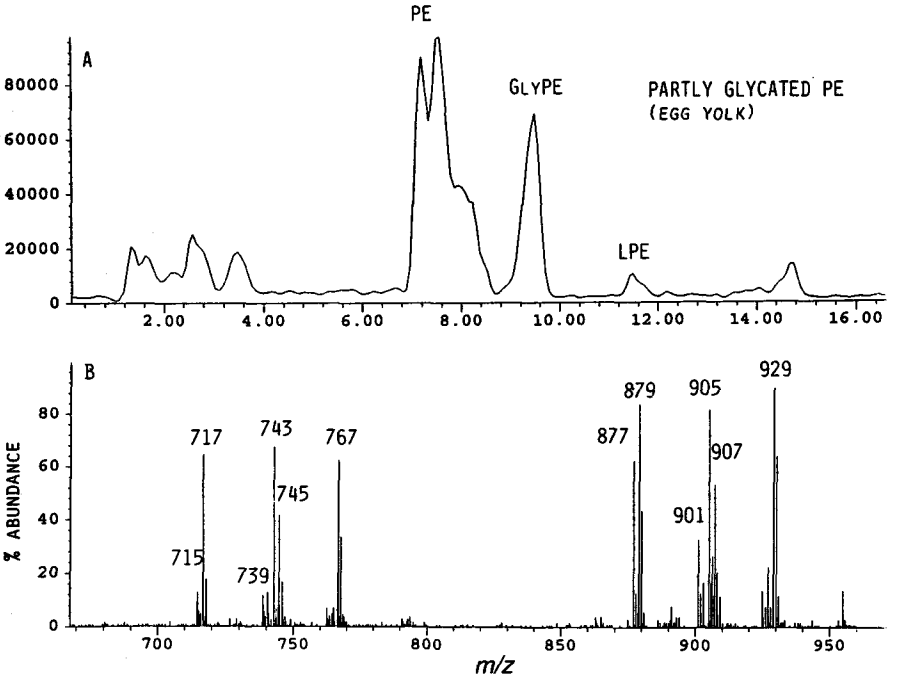


FIG. 5. Normal-phase liquid chromatography/mass spectrometry (MS) with electrospray of partially glucosylated egg yolk PE. A, total ion current profile; B, mass spectra averaged over the peak of nonglucosylated PE and of glucosylated PE. High-performance liquid chromatography and MS conditions are given in Figure 2 and in the Experimental Procedures section.

systems allowed effective extraction and chromatography of the cyanoborohydride reduction products of the glucosylated aminophospholipids. In the present study, we noted that alkaline solvent systems were best suited for the recovery of the glucosylated aminophospholipids from *in vitro* incubations.

The effect of pH was investigated only over a limited range, keeping close to physiological conditions. This was done in order to approximate the nonenzymatic *in vivo* formation of Schiff bases between the aldehyde form of glucose and the aminophospholipids, and to avoid subsequent transformations. More alkaline conditions (pH > 7.4) were not used because of potential hydrolysis of the lipid ester bonds during prolonged incubation. The alkaline HPLC solvent system and the soft ESI allowed us to demonstrate the glucosylation of both PE and PS in the presence of glucose concentrations found in severe diabetes and hyperglycemia. Such glucose concentrations have been shown to cause membrane damage and cell death of cultured pericytes, endothelial cells, kidney cells, retinal rods, and red blood cells (13–16). Incubation of intact red blood cells with increasing concentrations of glucose under isotonic conditions and pH 7.4 led to glucosylation of largely PE, which we hope to describe in greater detail elsewhere, along with the *in vivo* glucosylation of red blood cell aminophospholipids (A. Ravandi and A. Kuksis, unpublished results). In other unpublished studies, we have demonstrated that glucosylation of PE and PS results in increased accumulation of lipid ester hydroperoxides and core aldehydes in the aminophospholipids. It is not known whether this is due to increased susceptibility of the glucosylated aminophospholipids to peroxidation or to an interference of the glucosylated base structure with the removal of the hydroperoxy fatty acids from the phospholipid molecules by phospholipases A_2. Phospholipase A_2 is believed to be involved in cleavage of oxidized fatty acids as part of oxidation damage repair mechanism in cell membranes (17,18). The availability of the glucosylated standards along with well-defined working conditions for the isolation of these structures should allow an assessment of the natural distribution of the glucosylated aminophospholipids in normal and in pathological tissues. Comparable ESI spectra of nonglucosylated aminophospholipids recently have been reported (19,20).

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council of Canada, Ottawa, and the Heart and Stroke Foundation of Ontario, Toronto, Canada.

REFERENCES

1. Bucala, R., and Cerami, A. (1992) Advanced Glycosylation, Chemistry, Biology and Implications for Diabetes and Aging, *Adv. Pharmacol. 23*, 1–34.
2. Njoroge, F.G., and Monnier, V.M. (1989) The Chemistry of the Maillard Reaction Under Physiological Conditions: A Review, *Progr. Clin. Biol. Res. 304*, 85–107.
3. Bucala, R., Makita, Z., Koschinsky, T., Cerami, A., and Vlasaka, H. (1993) Lipid Advanced Glycosylation: Pathway for Lipid Oxidation *in vivo*, *Proc. Natl. Acad. Sci. USA 90*, 6434–6438.
4. Skipski, V.P., Peterson, R.F., and Buckley, M. (1964) Quantitative Analysis of Phospholipids by Thin-Layer Chromatography, *Biochem. J. 90*, 374–378.
5. Rose, H.G., and Oaklander, M. (1965) Improved Procedure for the Extraction of Lipids from Human Erythrocytes, *J. Lipid Res. 6*, 428–431.

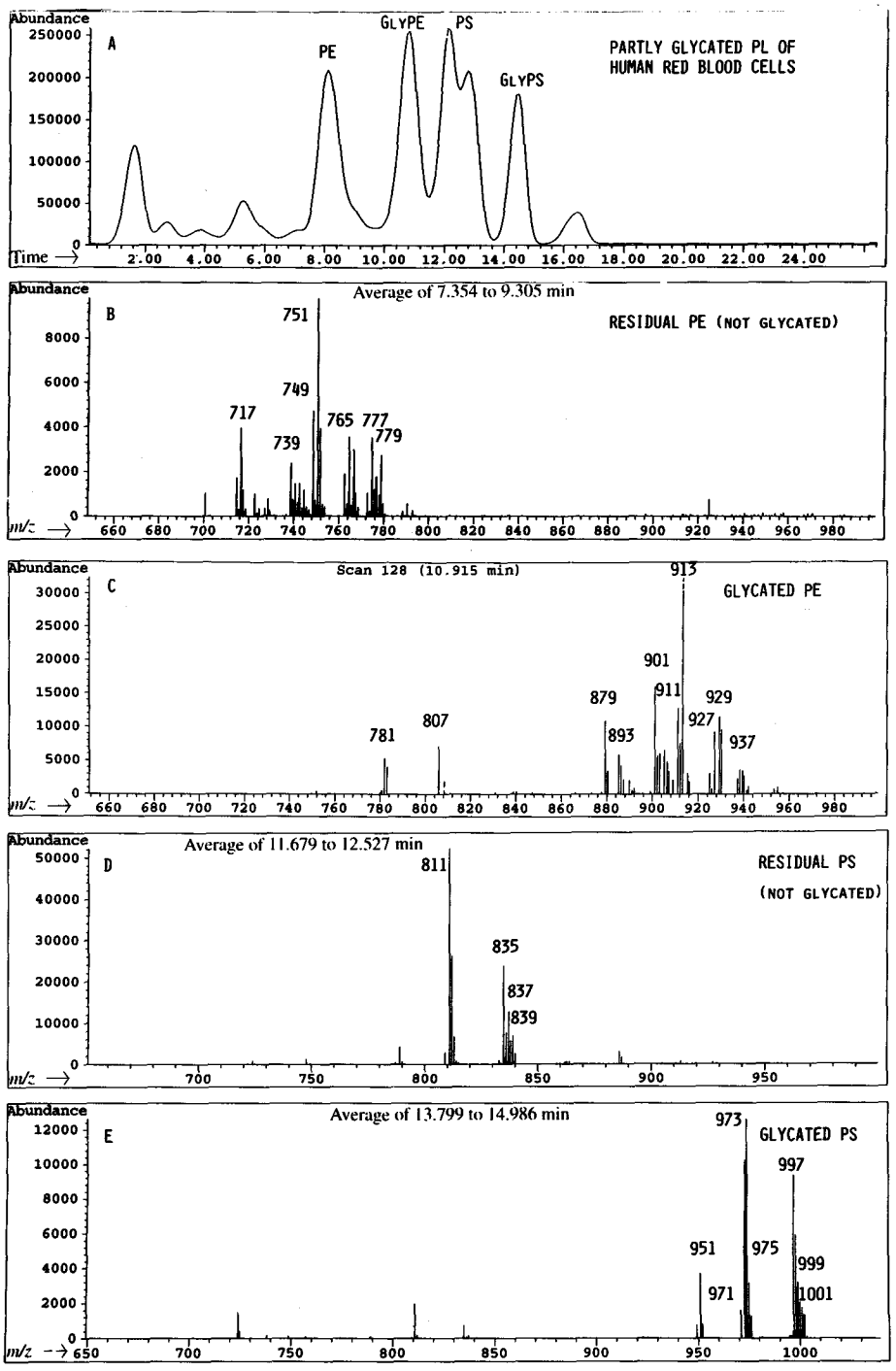


FIG. 6. Normal-phase liquid chromatography/mass spectrometry (MS) with electrospray of partially glucosylated aminophospholipids of human red blood cells. A, total ion current profile; B, mass spectra of nonglucosylated PE; C, mass spectra of glucosylated PE; D, mass spectra of nonglucosylated PS; E, mass spectra of glucosylated PS. LC/MS conditions are given in Figure 4 and in the Experimental Procedures section. Abbreviations as in Figure 1.

6. Rouser, G., Kritchevsky, G., Simon, G., and Nelson, G.J. (1967) Quantitative Analysis of Brain and Spinach Leaf Lipids Employing Silicic Acid Column Chromatography and Acetone for Elution of Glycolipids, *Lipids 2*, 37–40.
7. Myher, J.J., Kuksis, A. and Pind, S. (1989) Molecular Species of Glycerophospholipids and Sphingomyelins of Human Plasma: Comparison to Red Blood Cells, *Lipids 24*, 408–418.
8. Becart, J.T., Chevallier, C., and Bisse, J.P. (1990) Quantitative Analysis of Phospholipids by HPLC with a Light Scattering Evaporating Detector, *J. High Resol. Chromatogr. 13*, 126–129.
9. Jain, S.K. (1989) Hyperglycemia Can Cause Membrane Lipid Peroxidation and Osmotic Fragility in Human Red Blood Cells, *J. Biol. Chem. 264*, 21340–21345.
10. Kuksis, A. (1992) Yolk Lipids, *Biochim. Biophys. Acta 1124*, 205–222.
11. Myher, J.J., Kuksis, A., and Pind, S. (1989) Molecular Species

TABLE 1
Molecular Species of Aminophospholipids of Human Red Blood Cells Following Exposure to 50-mM Glucose[a]

Molecular species	Diacyl PE		Diacyl GlyPE		Alk-acyl PE		Alk-acyl Gly PE	
	$[M-H]^-$	%	$[M-H]^-$	%	$[M-H]^-$	%	$[M-H]^-$	%
PE								
16:0–18:1	717	14.7	879	15.3				
16:0–18:2	715	5.2	877	4.7				
18:0–18:1	745	2.8	907	1.3	729	2.3	891	1.1
16:0–20:4	739	15.3	901	13.9	723	7.1	885	6.8
18:1–18:1	743	5.8	905	6.4				
18:1–18:2	741	9.7	903	11.7	725	11.1	887	5.3
16:0–20:5	737	0.4	897	0.2	721	0.1	883	0.4
18:0–20:4	767	16.9	929	19.8	751	35.8	913	31.6
16:0–22:4	767		929		751		913	
18:1–20:3	767		929		751		913	
18:0–20:3	769	4.3	931	5.8	753	0.1	915	2.1
18:1–20:4	765	13.2	927	10.2	749	15.7	911	21.7
18:0–20:5	765		927		749		911	
16:0–22:5	765		927		749		911	
16:0–22:6	763	7.6	925	9.4	747	1.4	909	1.6
18:2–20:4	763		925		747		909	
18:2–20:5	793	0.8	955	0.7	777	9.9	939	13.7
18:1–22:4	793		955		777		939	
18:0–22:6	791	2.1	953	0.1	775	11.9	937	12.2
18:1–22:6	789	0.8	951	1.3	773	3.4	935	3.1
PS								
	PS		Gly PS					
	$[M-H]^-$	%	$[M-H]^-$	%				
18:0–18:1	789	6.6	951	8.3				
18:0–20:4	811	50.6	973	45.6				
16:0–22:4	811		973					
18:1–20:3	811		973					
18:0–20:3	813	7.3	975	10.3				
18:1–20:4	809	2.9	971	3.6				
16:0–22:5	809		971					
18:0–20:5	884		971					
18:0–22:5	837	9.1	999	12.6				
18:0–22:4	839		1001					
18:0–22:6	835	22.4	997	17.2				
18:1–22:6	833	1.1	995	0.8				

[a]Diacyl phosphatidylethanolamine (PE), 20.2%; diacyl glucosylated (Gly) PE, 24.5%; alkenylacyl (alk-acyl) PE, 24.0; alkenylacyl GlyPE, 31.1%; phosphatidylserine (PS), 48.6%; GlyPS, 51.3%.

of Glycerophospholipids and Sphingomyelins of Human Erythrocytes, *Lipids 24,* 396–407.
12. Bunn, H.F. and Higgins, P.J. (1981) Reaction of Monosaccharides with Proteins: Possible Evolutionary Significance, *Science 213,* 222–224.
13. Li, W., Shen, S., Khatami, M., and Rockey, J.H. (1984) Stimulation of Retinal Capillary Perycyte Protein and Collagen Synthesis in Culture by High-Glucose Concentration, *Diabetes 33,* 785–789.
14. Mandel, S.S., Shin, D.H., Newman, B.L., Lee, J.H., Lupovitch, A., and Draker, G.H. (1983) Glycosylation *in vivo* of Human Lens Capsule and Diabetes Mellitus, *Biochem. Biophys. Res. Commun. 117,* 51–56.
15. Cagliero, E., Maiello, M., Boeri, D., Roy, S. and Lorenzi, M. (1988) Increased Expression of Basement Membrane Components in Human Endothelial Cells Cultured in High Glucose, *J. Clin. Invest. 82,* 735–738.
16. Jones, R.C., and Peterson, C.M. (1981) Hematologic Alterations in Diabetes Mellitus, *Am. J. Med. 70,* 339–352.
17. Saglo, M.G., Corongiu, F.P., and Sevanian, A. (1992) Peroxidation and Phospholipase A_2 Hydrolytic Susceptibility of Liposomes Consisting of Mixed Species of Phosphatidylcholine and Phosphatidylethanolamine, *Biochim. Biophys. Acta 927,* 131–190.
18. Van den Berg, J.J.M., Op den Kamp, J.A.F., Lubin, B.H., and Kuipers, F.A. (1993) Conformational Changes in Oxidized Phospholipids and Their Preferential Hydrolysis by Phospholipase A. A Monolayer Study, *Biochemistry 32,* 4962–4967.
19. Myher, J.J., Kuksis, A., Ravandi, A., and Cocks, N. (1994) Normal-Phase Liquid Chromatography/Mass Spectrometry with Electrospray for Sensitive Detection of Oxygenated Glycerophospholipids, *INFORM 5,* 478–479.
20. Han, X. and Gross, R.W. (1994) Electrospray Ionization Mass Spectrometric Analysis of Human Erythrocyte Plasma Membrane Phospholipids, *Proc. Natl. Acad. Sci. USA, 91,* 10635–10639.

[Received February 17, 1995, and in final revised form June 27, 1995; Revision accepted August 16, 1995]

Structural Importance of the *cis*-5 Ethylenic Bond in the Endogenous Desaturation Product of Dietary Elaidic Acid, *cis*-5,*trans*-9 18:2 Acid, for the Acylation of Rat Mitochondria Phosphatidylinositol

Robert L. Wolff

I.S.T.A.B., Laboratoire de Lipochimie Alimentaire, Université Bordeaux 1, Talence, France

ABSTRACT: When rats were fed elaidic (*trans*-9 18:1) acid at a high load in diets that were otherwise marginally or almost completely deficient in linoleic (*cis*-9,*cis*-12 18:2) acid, elaidic acid was desaturated to *cis*-5,*trans*-9 18:2 acid. This polymethylene-interrupted acid was then incorporated into most phospholipids from rat mitochondria, cardiolipin being an exception. Its level of esterification in phospholipids followed the increasing order: phosphatidylethanolamine < phosphatidylcholine < phosphatidylinositol (PI). The content of *cis*-5,*trans*-9 18:2 acid decreased in organs in the order liver > kidney > heart. The levels of *cis*-5,*trans*-9 18:2 acid increased in mitochondria phospholipids as the level of linoleic acid was lowered in the diet. In liver mitochondria PI, it reached 16% of total fatty acids. After hydrolysis of liver mitochondria PI with *Naja naja* phospholipase A_2, we observed that elaidic acid was essentially esterified to position 1 at the expense of saturated acids, whereas *cis*-5,*trans*-9 18:2 acid was exclusively esterified to position 2, along with 20:3n-9 and 20:4n-6 acids. As a consequence, the sums of saturated and *trans*-9 18:1 acids on the one hand, and of 20:3n-9, 20:4n-6, and *cis*-5,*trans*-9 18:2 acids on the other hand, remained fairly constant in liver mitochondria PI (*ca.* 55 and 30%, respectively). Because *trans*-9 18:1 and *cis*-5,*trans*-9 18:2 acids differ only by the *cis*-5 ethylenic bond, which is also present in 20:3n-9 and 20:4n-6 acids, this distribution pattern indicates that the *cis*-5 double bond, rather than any other ethylenic bond, may be of major structural importance for channeling fatty acids to position 2 of PI.

Lipids 30, 893–898 (1995).

Little is known about the specificity of acylases that ensure the homeostasis of membrane phospholipid fatty acids *in vivo*. There are at least three types of acylases—acyl-CoA acyltransferase, CoA-dependent, and CoA-independent transacylases. To our knowledge, few data have been published on the structural parameters of fatty acids that are recognized by such phospholipid acylating enzymes. For example, it has been suggested that acylases which are specific for the 1(1″) positions of cardiolipin (CL) esterify only fatty acids having at least one *cis*-9 double bond, with no other *cis* double bonds between this position and the carboxylic group (1). In the case of highly polyunsaturated fatty acids such as 20:4n-6 (all *cis*-5,8,11,14 20:4) or 22:6n-3 (all *cis*-4,7,10,13,16,19 22:6) acids, the question that arises is: Which parts of the molecules, which double bonds, are specifically identified by acylases?

The problem seems to be somewhat simplified in phosphatidylinositol (PI), because this phospholipid essentially contains 20:4n-6 acid as a polyunsaturated fatty acid, with practically no 22:6n-3 acid. The situation in this particular case is thus limited to establishing which part of the arachidonic acid molecule is specifically identified by PI-acylating enzymes. It is clear that the *cis*-14 double bond is not essential, because 20:4n-6 acid is easily replaced by 20:3n-9 (all *cis*-5,8,11 20:3) acid, which is devoid of this bond, when the precursor of 20:4n-6 acid, linoleic acid, is depleted in the diet and, therefore, in the cell. Moreover, it was recently shown that 20:4n-6 acid could also be replaced in PI by the rare all *cis*-5,11,14 20:3 acid (2). As this acid lacks the *cis*-8 double bond, it can be deduced that this bond is not essential for the recognition by PI-specific acylating enzymes. So, there remain two double bonds, the *cis*-5 and the *cis*-11, that may be of some structural importance for the specificity of PI-acylating enzymes. Because PI contains only trace amounts of *cis*-11 18:1 (*cis*-vaccenic) acid, we hypothesized that the *cis*-5 double bond might be of major importance. However, to decide which of these double bonds is significant, it is necessary to test fatty acids with isolated *cis*-5 or *cis*-11 double bonds.

Fatty acids containing an isolated *cis*-5 double bond exist, but they are not easily available in sufficient quantities for dietary experiments. For example, pine seed oil contains *cis*-5,*cis*-9,*cis*-12 18:3 acid (3), whereas meadowfoam seed oil is rich in *cis*-5 20:1 acid (4). In the present study, we took advantage of a surprising property of elaidic (*trans*-9 18:1) acid —it can be desaturated to *cis*-5,*trans*-9 18:2 acid either *in vitro* or *in vivo* in the rat, provided the diet is either marginally or completely deficient in linoleic acid (5,6). This uncom-

*Address correspondence at I.S.T.A.B., Laboratoire de Lipochimie Alimentaire, Université Bordeaux 1, Allée des Facultés, 33405 Talence Cedex, France.

Abbreviations: CL, cardiolipin; DCF, 2′,7′-dichlorofluorescein; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

mon polymethylene-interrupted acid contains an isolated *cis*-5 double bond, and because the *trans*-9 double bond is sterically similar to a single bond (see the Discussion section), the *cis*-5,*trans*-9 18:2 acid is geometrically equivalent to a *cis*-5 18:1 acid. Consequently, we fed rats diets rich in elaidic acid and poor in linoleic acid, and followed the metabolic fate of the endogenously synthesized *cis*-5,*trans*-9 18:2 acid. For this purpose, we analyzed the fatty acids of all individual phospholipids from mitochondria of different rat organs (liver, kidneys, heart), with particular attention to PI. This quantitatively minor phospholipid represents only 3–8% (on a phosphorus basis) of total mitochondria phospholipids, depending on the organ.

MATERIALS AND METHODS

Animals and diets. Three groups of 12 weanling male Wistar rats (mean weight: 45 ± 2 g; 21-day-old) were raised for one month on a basic fat-free diet (UAR, Villemoisson-sur-Orge, France) supplemented with 10% (w/w) of a synthetic triglyceride (trielaidin) mixed with a variable amount of soybean oil (in order to provide a small percentage of linoleic and α-linolenic acids). The fatty acid compositions of the fat supplements are summarized in Table 1. The elaidic acid content was kept almost constant (from 64.5 to 69.0%), whereas that of linoleic acid varied from 0.3 to 7.1% of total fatty acids. In addition to these three groups of rats, ten weaned rats and six young adult rats (*ca.* 150 g) that were reared on a standard complete diet were also used in the present study.

Preparation of mitochondria. At the end of the feeding periods, the rats were decapitated and their liver, kidneys, and heart were excised. The organs were washed with ice-cold buffer (10 mM Tris, 0.25 M sucrose, 2 mM EDTA, pH 7.2), cut into small pieces, pooled, and rinsed with the buffer. Eight volumes of the buffer were added (Bioblock Scientific, Strasbourg, France) and the mixture was homogenized in a Potter homogenizer. For hearts only, type VIII protease from *Bacillus subtilis* (0.8 mg protease/g of heart tissue; Sigma Chemical Company, St. Louis, MO) was added to the homogenate. Mitochondria were then prepared by successive centrifugations (7) and were finally resuspended in a minimum volume of buffer containing 0.25 M sucrose and 10 mM Tris (pH 7.2). All operations were conducted at 4°C.

TABLE 1
Fatty Acid Composition (wt% total fatty acids) of the Fat Supplement in the Experimental Diets

Fatty acids	Diet A	Diet B	Diet C
16:0	1.1	0.3	0.7
18:0	0.6	0.3	0.3
cis-9 18:1	24.9	27.8	29.0
trans-9 18:1	64.5	69.0	67.5
18:2n-6	7.1	2.4	0.3
18:3n-3	0.7	0.1	<0.1
Others	1.1	0.9	2.1

Extraction of lipids. Seven volumes of methanol were added to the mitochondria suspensions, followed by 14 vol of chloroform. The suspensions were stirred for 30 min at room temperature and filtered on a paper filter into a rotary evaporator flask. Solvents were evaporated under reduced pressure, and lipids were dissolved in pure chloroform. The chloroform solution of lipids was transferred and filtered in a tube. Chloroform was evaporated under a stream of N_2, and lipids were dissolved in chloroform/methanol (2:1, vol/vol). After washing according to the procedure of Folch *et al.* (8), the lower layer was filtered and the solvents were evaporated. Lipids were stored at −20°C in chloroform/methanol (2:1, vol/vol) under N_2 in the presence of 0.02% 2,6-di-*tert*-butyl-4-methylphenol as an antioxidant.

Phospholipid isolation. Phospholipids were separated by thin-layer chromatography (TLC) on 0.3-mm thick silica gel H (Kieselgel H 60; Merck, Darmstadt, Germany) laboratory-coated plates using the solvent mixture chloroform/methanol/water (65:25:4, by vol). Phospholipid bands [phosphatidylcholine (PC) plus PI (PC–PI); phosphatidylethanolamine (PE); CL] were visualized under ultraviolet light after lightly spraying the plates with a 0.2% (wt/vol) solution of 2′,7′-dichlorofluorescein (DCF) in 96% ethanol. The bands containing PE and CL were scraped off and transferred into Teflon-lined screw-capped tubes for transmethylation of their fatty acids. The gel band containing PC–PI was transferred into a centrifuge tube, wetted with a few drops of distilled water, and the phospholipids were extracted twice with chloroform/methanol (2:1, vol/vol). The solvent was evaporated under a stream of N_2 at 30°C and the residue dissolved in chloroform/methanol (2:1, vol/vol). This solution was washed with 0.2 vol of a 0.8% KCl aqueous solution, and several times with portions of Folch upper phase (7) until no fluorescence due to the DCF could be detected under ultraviolet light. The solvent was evaporated, and the phospholipids were dissolved in a small volume of chloroform for further processing.

The preceding extract of mixed PC and PI was fractionated a second time by TLC with the solvent mixture butanol/acetic acid/water (40:20:20, by vol). After evaporation of the solvents from the plates with a stream of N_2, phospholipids were detected, and the gel was scraped off and transferred into Teflon-lined screw-capped tubes for transmethylation. In one instance, PI was eluted from the silica gel and purified as described previously for further hydrolysis with phospholipase A_2.

Hydrolysis of PI with phospholipase A_2. Hydrolysis of the ester bonds at position 2 of *sn*-glycerol of PI from liver mitochondria (purified as described previously) with phospholipase A_2 from *Naja naja* venom (Sigma Chemical Company) was performed according to a previously published method (9). The hydrolysis appeared to be complete after an incubation period of 2 h. LysoPI was separated from the free fatty acids from position 2 by TLC on silica gel H-coated plates developed with chloroform/methanol/water (65:25:4, by vol). After visualization of the bands, the gel was scraped off and

transferred into Teflon-lined screw-capped tubes for methyl ester preparation.

Fatty acid methyl ester (FAME) preparation and analysis. FAME from PC, PI, PE, and CL were prepared essentially according to Morrison and Smith (10). A methanolic solution (1–1.5 mL) of BF_3 (12%, wt/vol; Fluka, Buchs, Switzerland) was added to individual phospholipids in the presence of silica gel, and methylation was performed at 90°C for 30 min. FAME were analyzed by gas–liquid chromatography (GLC) using Varian (Palo Alto, CA) 1400 and 940 chromatographs equipped with flame-ionization detectors. Analyses were carried out with 300 x 0.32 cm or 600 x 0.32 cm stainless-steel columns packed, respectively, with 7% BDS (stationery phase; Supelco France, Saint-Gormain-en-Laye, France) on 100–120 mesh gas-chrom Q, at 185°C, or 15% OV-275 (stationary phase; Supelco France) on 80–100 mesh chromosorb P-DMCS, at 215°C. Peaks were identified by comparison of their retention times relative to those of commercially-available standards. Peak identification was supported by GLC analysis of FAME after fractionation by TLC on layers of silica gel H containing 5% silver nitrate (by weight). These plates were developed twice with hexane/diethyl ether/acetic acid (94:4:2, by vol) (11). FAME prepared from *Taxus baccata* L. seed oil, which contains *cis*-5,*cis*-9 18:2 acid (12), were used as a secondary standard on the BDS column for the location of the polymethylene-interrupted diene *cis*-5,*trans*-9 18:2 acid. Peak areas and percentages were calculated by a Shimadzu ICR-1B integrator (Shimadzu, Tokyo, Japan).

RESULTS

After a one-month feeding period on the elaidic acid-rich diets, the *cis*-5,*trans*-9 18:2 acid was esterified to PI, PC, and PE (except in PE from heart mitochondria of rats fed diet A), but practically not in CL (Fig. 1). This occurred in all three organs (liver, kidneys, and heart). Independently of the organ or the level of linoleic acid in the diet, the highest percentages of *cis*-5,*trans*-9 18:2 acid were observed in PI, followed by PC and then by PE. As the content of linoleic acid was lowered in the diet, the rate of incorporation of *cis*-5,*trans*-9 18:2 acid increased in mitochondria PI, PC, and PE from the three organs. A maximum value of 16% was reached in PI from liver mitochondria of rats fed the diet with the minimum amount of linoleic acid (0.3%; diet C). For each of the three phospholipids, the percentage of *cis*-5,*trans*-9 18:2 acid decreased with the organ in the order: liver > kidneys > heart (Fig. 1). Following hydrolysis by phospholipase A_2 of PI from liver mitochondria of rats fed the diet with the lowest amount of linoleic acid (diet C), we observed that the *cis*-5,*trans*-9 18:2 acid was exclusively located at the 2-position, together with 20:3n-9 and 20:4n-6 acids (Fig. 2). On the other hand, elaidic acid was essentially only esterified to position 1 together with saturated acids, mainly stearic acid. We also found an unknown fatty acid in a small amount at position 2 of PI (X in Fig. 2). From its behavior during GLC, this acid should have 20 carbon atoms. It migrated along with *cis*-5,*trans*-9 18:2 acid upon Ag–TLC fractionation, which sug-

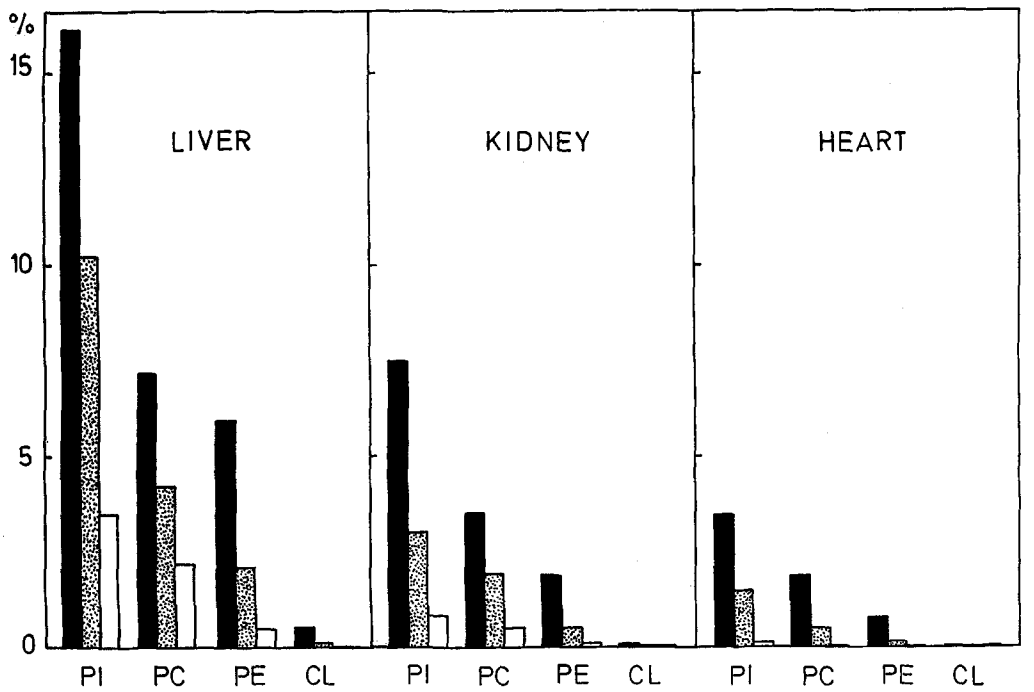


FIG. 1. Levels of *cis*-5,*trans*-9 18:2 acid in phospholipids of mitochondria from different organs of rats fed diets containing an almost constant percentage of elaidic acid (*ca.* 67% of total fatty acids in the fat supplement) with decreasing levels of linoleic acid after a one-month feeding period. Solid bars: 0.3% of linoleic acid (diet C); dotted bars, 2.4% (diet B); open bars, 7.1% (diet A). Results are expressed as weight percentages of *cis*-5,*trans*-9 18:2 acid relative to total fatty acids in a given phospholipid and are the means of at least two analyses. PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin.

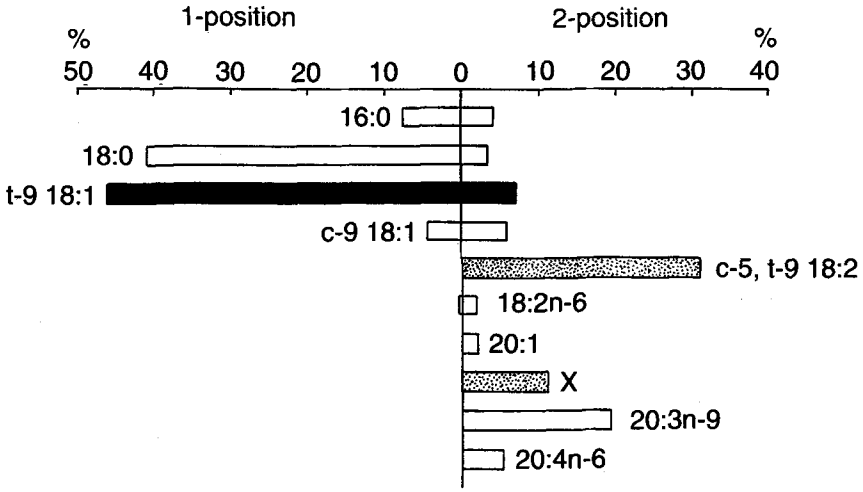


FIG. 2. Distribution of fatty acids between the 1- and 2-positions of phosphatidylinositol purified from liver mitochondria of rats fed for one month a diet containing 10% of fat, of which 68 and 0.3% were elaidic and linoleic acids respectively, (diet C). Results are expressed as weight percentages of fatty acids esterified to each position. X, unknown fatty acid. c, *cis*; t, *trans*.

gests that the two acids have analogous structural features. As this unknown acid was absent from liver PI of rats fed a fat-free diet, devoid of elaidic acid (results not shown), we can hypothesize that X was derived from elaidic acid. When comparing the fatty acid compositions of liver PI from rats fed the three experimental diets with those of liver PI from weaning or young adult rats reared on a complete standard diet, one can establish that the sums of saturated and elaidic acids on the one hand, and of *cis*-5,*trans*-9 18:2 acid (plus the unknown acid), 20:3n-9 and 20:4n-6 acids on the other hand, did not vary much (*ca.* 55 and 30%, respectively) (Fig. 3). In two instances (diets A and B), we followed the time-course evolution of fatty acids in liver mitochondria PI (Fig. 4). Although elaidic acid reached its maximum value after only one week in both cases, the increase of *cis*-5,*trans*-9 18:2 acid was much slower and almost paralleled that of 20:3n-9 acid for the replacement of 20:4n-6 acid. The behavior of the unknown acid followed that of *cis*-5,*trans*-9 18:2 acid. The rate of incorporation of *cis*-5,*trans*-9 18:2 acid increased as the level of linoleic acid in the diet was lowered.

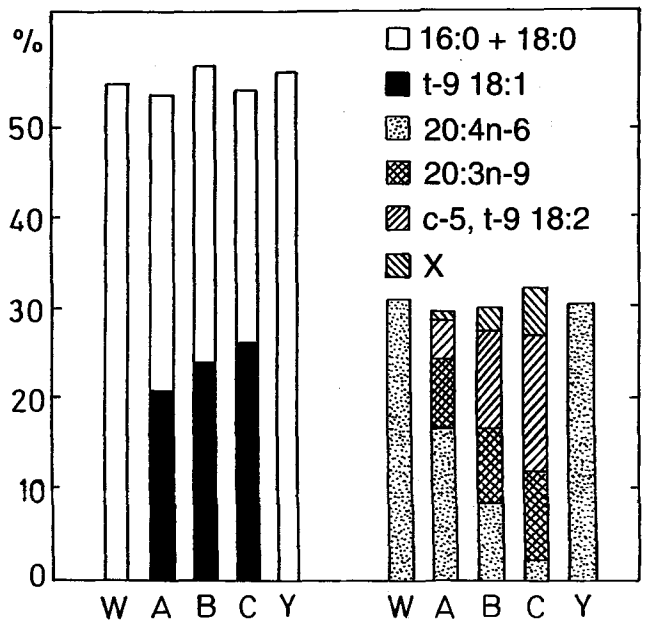


FIG. 3. Fatty acid distributions (main components) in phosphatidylinositol of liver mitochondria from rats fed for one month diets containing 10% of fat containing *ca.* 67% elaidic acid and a variable amount of linoleic acid (see Table 1 for the detailed fatty acid compositions of the diets). Data for weaning rats (W) and young adult rats fed a standard complete diet (Y) are also included. X, unknown fatty acid. c, *cis*; t, *trans*; A, B, C, diets A, B, and C.

DISCUSSION

From the phospholipase A_2 experiment, it is clear that the presence of the *cis*-5 ethylenic bond in *cis*-5,*trans*-9 18:2 acid is fundamental for channeling this acid to the 2-position of PI. Elaidic acid, which lacks this double bond, was essentially only esterified at the 1-position, where it replaced saturated acids. This confirms our previous results, which showed that elaidic acid should be considered as geometrically equivalent to a saturated fatty acid, at least for the esterification of phospholipids. It replaced saturated alkyl chains in PI, PC, and PE (13) and saturated alkenyl chains (after its reduction to the corresponding alcohol) in plasmalogens (14,15). On the other hand, it was hardly incorporated at all in CL (16), which is naturally almost devoid of saturated acids (1,17). The fact that a *trans* double bond should be considered as geometrically equivalent to a single bond is not limited to the *trans*-9 double bond in elaidic acid. For example, *trans*-5,*cis*-9,*cis*-12 18:3 and *cis*-9,*cis*-12,*trans*-15 18:3 acids were mainly incorporated in CL (2,18), which otherwise has a particular affin-

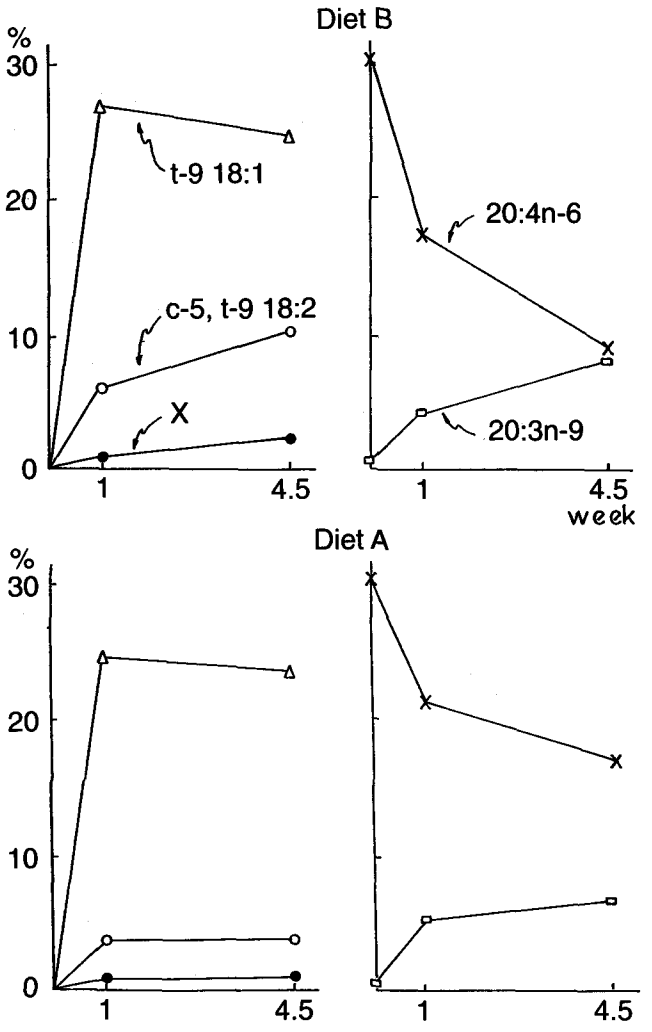


FIG. 4. Time-course evolution of some fatty acids in phosphatidylinositol from liver mitochondria of rats fed diets containing 10% of fat, in which elaidic and linoleic acids accounted for 69.0 and 2.4%, respectively (diet B), or 64.5 and 7.1%, respectively (diet A). X, unknown fatty acid; c, *cis*; t, *trans*.

ity for *cis*-9,*cis*-12 18:2 acid (14). In these two cases, the *trans*-5 and *trans*-15 double bonds were apparently perceived like single bonds by CL-acylating enzymes. The *trans*-5,*cis*-9,*cis*-12 18:3 and *cis*-9,*cis*-12,*trans*-15 18:3 acids thus behaved like *cis*-9,*cis*-12 18:2 acid and were preferentially channeled to CL. In an unpublished experiment, we observed that *cis*-5,*cis*-8,*cis*-11,*trans*-14 20:4 acid, which is endogenously derived from dietary *cis*-9,*trans*-12 18:2 acid (19,20), was incorporated mainly into liver PI, suggesting that the *trans*-14 20:4n-6 acid behaves like 20:3n-9 acid and consequently, that the *trans*-14 double bond is also equivalent to a single bond. This last observation also supports the conclusion that a *cis*-14 double bond is not necessary for a fatty acid to be esterified in PI.

The introduction of a *cis*-5 ethylenic bond in elaidic acid considerably modifies its properties. It is not handled by acylating enzymes like a saturated acid, but like a *cis*-unsaturated acid with a high affinity for PI and, more precisely, for its 2-position. Among phospholipids, PI contains the highest level of 20:4n-6 acid when 18:2n-6 acid is not limited in the diet as in a complete standard diet (*ca.* 30% of total fatty acids, or even more). On the other hand, 20:4n-6 acid is very low in CL. The 20:4n-6 acid contents in PC and PE are intermediate between these two extremes. So, it is tempting to hypothesize that *cis*-5,*trans*-9 18:2 acid follows the same route as 20:4n-6 acid for acylation into phospholipids. It is not incorporated in CL, and it reaches its maximum value in PI with intermediate levels in PC and PE. This hypothesis is supported by the kinetics of incorporation of *cis*-5,*trans*-9 18:2 acid into liver mitochondria PI (Fig. 4). The esterification of elaidic acid is very rapid, the maximum being reached in only one week, or perhaps less. Due to the shortness of this period, it appears that saturated acids in PI are quickly exchanged through deacylation–reacylation with elaidic acid rather than incorporated in *de novo*-synthesized molecules. On the other hand, the incorporation of *cis*-5,*trans*-9 18:2 acid in position 2 of PI was comparatively rather slow. There are at least two possible explanations for this phenomenon. First, the turn-over rate of 20:4n-6 acid might be slower than that of saturated acids in PI. Consequently, and because the precursor of 20:4n-6 acid was low in the diet, one may speculate that *cis*-5,*trans*-9 18:2 acid was mainly incorporated in *de novo*-synthesized PI molecules, in lieu of 20:4n-6 acid, which was depleted in the intracellular fatty acid pool, rather than exchanged with this acid. One may also hypothesize that the desaturation of elaidic acid by the Δ5 desaturase was a slow process, and that the accumulation of *cis*-5,*trans*-9 18:2 acid in the intracellular fatty acid pool was also slow and progressive. Moreover, *cis*-5,*trans*-9 18:2 acid might compete with 20:3n-9 acid, which is derived from oleic (*cis*-9 18:1) acid, for the replacement of 20:4n-6 acid in PI. Such conclusions cannot be drawn from the study of PC and PE fatty acids, because there is a concomitant decrease of both 20:4n-6 and 22:6n-3 acids in these phospholipids (results not shown).

Results obtained in the present study support the view that an acylase (or a group of enzymes involved in acylation) specific for PI exists that is also specific for fatty acids containing a *cis*-5 double bond. The 20:4n-6, 20:3n-9, all *cis*-5,11,14 20:3 and *cis*-5,*trans*-9 18:2 acids all share in common this double bond and present a higher affinity for PI than for any other phospholipid. We observed that the level of *cis*-5 double bond-containing fatty acids remains fairly constant in PI (Fig. 3). Because these acids are esterified to the 2-position of PI, this would imply that about 60% of PI molecules are substrate of the *cis*-5 double bond-specific acylating enzymes. The fatty acid profile in this population should thus only depend on the availability of the different *cis*-5 double bond-containing fatty acids in the intracellular fatty acid pool. Under conditions where linoleic acid is not limiting, 20:4n-6 acid is the main *cis*-5 double bond-containing acid in this pool. According to the concept developed in this study, it is probably recognized by the specific acylase through its *cis*-5

double bond, which thus appears to be the minimum structural requirement for this recognition.

ACKNOWLEDGMENTS

This study was performed in Prof. B. Entressangles's laboratory, and Dr. N.A. Combe provided skillful technical assistance. Trielaidin was a kind gift from the Astra-Calvé company.

REFERENCES

1. Wolff, R.L., and Entressangles, B. (1991) Compositional Changes of Fatty Acids in the 1(1″)- and 2(2″)-Positions of Cardiolipin from Liver, Heart and Kidney Mitochondria of Rats Fed a Low-Fat Diet, *Biochim. Biophys. Acta 1082*, 136–142.
2. Berger, A., and German, J.B. (1991) Extensive Incorporation of Dietary Δ-5,11,14 Eicosatrienoate into the Phosphatidylinositol Pool, *Biochim. Biophys. Acta 1085*, 371–376.
3. Takagi, T., and Itabashi, Y. (1982) *Cis*-5-Olefinic Unusual Fatty Acids in Seed Lipids of Gymnospermae and Their Distribution in Triacylglycerols, *Lipids 17*, 716–723.
4. Padley, F.B., Gunstone, F.D., and Harwood, J.L. (1994) Occurrence and Characteristics of Oils and Fats, in *The Lipid Handbook* (Gunstone, F.D., Harwood, J.L., and Padley, F.B., eds.) pp. 47–146, Chapman and Hall, London.
5. Lemarchal, P., and Bornens, M. (1968) Désaturation de l'Acide Elaïdique par le Foie de Rat *in vitro*, *Bull. Soc. Chim. Biol. 50*, 195–217.
6. Munsch, N. (1970) Influence de l'Ingestion de Triélaïdine sur la Composition en Acides Gras des Lipides Hépatiques et Plasmatiques de Rat, *Ann. Nutr. Alim. 24*, 21–40.
7. Blond, J.P., Clouet, P., and Lemarchal, P. (1975) Oxydation de l'Acide Erucique et de l'Erucyl-CoA par les Mitochondries Isolées de Coeur de Rat. Comparaison à l'Acide Oléique, *Biochimie 57*, 361–367.
8. Folch, J., Lees, M., and Sloane-Stanley, G.M. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
9. Wolff, R.L., and Combe, N. (1985) Cardiolipides: Purification et Hydrolyse Enzymatique Rapide par la Phospholipase A_2, *Rev. Fr. Corps Gras 32*, 251–256.
10. Morrison, W.R., and Smith, L.M. (1964) Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Trifluoride–Methanol, *J. Lipid Res. 5*, 600–608.
11. Dudley, PA., and Anderson, R.E. (1975) Separation of Polyunsaturated Fatty Acids by Argentation Thin-Layer Chromatography, *Lipids 10*, 113–114.
12. Madrigal, R.V., and Smith, C.R., Jr. (1975) *Taxus baccata* Seed Oil: A New Source of *cis*-5,*cis*-9-Octadecadienoic Acid, *Lipids 10*, 502–504.
13. Wolff, R.L., and Entressangles, B. (1994) Steady-State Fluorescence Polarization Study of Structurally Defined Phospholipids from Liver Mitochondria of Rats Fed Elaidic Acid, *Biochim. Biophys. Acta 1211*, 198–206.
14. Wolff, R.L., Combe, N.A., and Entressangles, B. (1985) Modification of Alkenyl Chain Profile in Plasmalogens of Rat Heart Mitochondria by Dietary Trielaidin, *Lipids 20*, 367–372.
15. Wolff, R.L., Combe, N.A., and Entressangles, B. (1988) Evolution au Cours du Temps de la Composition en Chaines Alkényles et Acyles des Plasmalogènes de Mitochondries de Coeur et de Reins chez des Rats Ingérant de la Triélaïdine, *Reprod. Nutr. Develop. 28*, 603–615.
16. Wolff, R.L., Combe, N.A., and Entressangles, B. (1984) Incorporation et Elimination de l'Acide Elaïdique dans les Différents Phospholipides des Mitochondries ou de leurs Membranes chez le Rat. Influence de l'Organe et de l'Age, *Rev. Fr. Corps Gras 31*, 161–170.
17. Wolff, R.L., Combe, N.A., and Entressangles, B. (1985) Positional Distribution of Fatty Acids in Cardiolipin from 21-Day-Old Rats, *Lipids 20*, 908–914.
18. Wolff, R.L., Combe, N.A., Entressangles, B., Sebedio, J.-L., and Grandgirard, A. (1993) Preferential Incorporation of Dietary *cis*-9,*cis*-12,*trans*-15 18:3 Acid into Rat Cardiolipins, *Biochim. Biophys. Acta 1168*, 285–291.
19. Blank, M.L., and Privett, O.S. (1963) Studies of the Metabolism of *cis*,*trans* Isomers of Methyl Linoleate and Linolenate, *J. Lipid Res. 4*, 470–476.
20. Beyers, E.C., and Emken, E.A. (1991) Metabolism of *cis*,*trans* and *trans*,*cis* Isomers of Linoleic Acid in Mice and Incorporation into Tissue Lipids, *Biochim. Biophys. Acta 1082*, 275–284.

[Received May 16, 1995, and in final revised form July 17, 1995; Revision accepted July 27, 1995]

A Temperature-Sensitive Strain of *Histoplasma capsulatum* Has an Altered Δ^9-Fatty Acid Desaturase Gene

Silvana Gargano[a], Gustavo Di Lallo[a,1], George S. Kobayashi[b] and Bruno Maresca[a,b,*]

[a]International Institute of Genetics and Biophysics, CNR, 80125 Naples, Italy and [b]Washington University, School of Medicine, Department of Internal Medicine, Division of Infectious Diseases, St. Louis, Missouri 63110-1093

ABSTRACT: We have isolated and characterized the Δ^9-desaturase gene (*Ole1*), which codes for a key enzyme involved in regulating membrane fluidity in animal cells and microorganisms, from two strains of *Histoplasma capsulatum*, one that is temperature-tolerant (G217B) and the other temperature-susceptible (Downs). These pathogenic fungi are dimorphic in that they undergo a morphologic transition from the mycelial to yeast-like form when the temperature of incubation is switched from 25 to 37°C or when they infect a susceptible host. The coding sequences of the two genes, both containing an intron of 93 nucleotides, are virtually identical and analogous to the Δ^9-desaturase gene of *Saccharomyces cerevisiae* and those of the rat, mouse and human. *Ole1* transcription of the thermotolerant G217B and thermosensitive Downs strains is similar in yeast phase cells and during the temperature shift down from 34, 37, or 40 to 25°C (yeast-to-mycelia transition). Nevertheless, the Δ^9-desaturase gene is transcriptionally inactive in mycelia of G217B at 25°C while it is actively transcribed in the Downs strain at the same temperature. These results are in agreement with the finding that membranes of the Downs strain have a higher level of oleic acid. The differential expression of Δ^9-desaturase genes is discussed in relationship to differences in thermosensitivity in the fungal isolates and in regulating the level of expression of heat shock genes.

Lipids 30, 899–906 (1995).

The physical properties of lipids and the dynamic nature of membranes have important effects on the functions of many membrane-associated proteins. Homeoviscous adaptation, a common feature of all organisms that adapt to different temperature regimes, is generally accomplished by incorporation of different types of fatty acids into the membrane during phospholipid synthesis or by regulation of the degree of unsaturation of the fatty acids itself (1–3). By modifying the transition temperature of membrane phospholipids, homeoviscous adaptation of the membrane maintains a given fluid environment for enzyme function at any physiological temperature. To determine whether a correlation exists between membrane fluidity and adaptation to sudden and rapid changes in temperature shifts up, the heat shock response (4), we began a study on the regulation of genes responsible for unsaturation of fatty acids in two strains of *Histoplasma capsulatum*, a dimorphic fungal pathogen of humans, that have different temperature optima of heat shock gene transcription (5,6).

As a saprobic fungus in nature, *H. capsulatum* grows in a multicellular filamentous form, but when it infects a susceptible host it becomes a unicellular yeast (7). Under laboratory conditions, the transition between the multicellular and unicellular phases can be reversibly induced by shifting the temperature between 25°C (mycelia) and 37°C (yeast). The mycelial-to-yeast-like conversion is of particular interest since it is triggered by an increase in the temperature of incubation, and conversion to the yeast morphology is necessary for virulence (8,9). The capacity to grow in different morphological forms implies that the organism is capable of adapting to different living conditions by expressing phase-specific genes whose products are critical for the survival and colonization of a given environment (10). Several biochemical and molecular events related to the process of temperature adaptation, that occur during the mycelial-to-yeast phase transition, have been studied (5,10–12). Heat shock proteins are among those products that have been best characterized (13). Heat shock genes are induced very early during infection and play a critical role in adaptation to the new environment (10). Recently, we have shown that the capacity to induce a stress response (transcription of heat shock genes) is influenced by the fatty acids composition of cell membranes (14; Carratù, L., Franceschelli, S., Pardini, C.L., Kobayashi, G.S., Horvath, I., Vigh, L., and Maresca, B., manuscript in preparation). Furthermore, the ratio of saturated/unsaturated fatty acids determines the appropriate membrane fluidity at a given temperature and environment (1). We have shown that the temperature-susceptible avirulent Downs strain, altered in its saturated/unsaturated fatty acids ratio, has a different optimal temperature of heat shock gene expression, i.e., 34°C, compared to 37°C in the temperature-tolerant virulent strains such as G217B (5,6) and a different biochemical pattern during the mycelium-to-yeast conversion (10,12).

It is well known that the physical state of the bilayer of biological membranes is a function of lipid composition and

[1]Dipartimento di Biologia, Università di Roma "Tor Vergata," Via della Ricerca Scientifica, 00133 Roma, Italy.

*To whom correspondence should be addressed.

Abbreviations: ORF, open reading frame; PCR, polymerase chain reaction.

Copyright © 1995 by AOCS Press

that temperature acclimation causes changes in levels of acyl unsaturation. In the case of temperature shifts down, the introduction of additional unsaturated bonds disrupts the close packing of adjacent lipid chains to compensate for the ordering effects of cold (15,16). The mechanisms that control lipid biosynthesis are not yet clear but unquestionably involve changes in the activity of desaturases and acyltransferases. We postulated that the membrane is a primary target in the biological perception of temperature during rapid temperature changes and proposed that saturated/unsaturated fatty acids ratio and lipoprotein membrane complexes are involved in the perception of temperature changes and that under conditions of heat shock, disturbance of membrane physical state causes transduction of a signal that induces transcription of heat shock genes.

In animal and fungal cells, unsaturated fatty acids are formed by fatty acid desaturases which are lipophilic microsomal enzymes (17). The Δ^9-desaturase gene product is the major enzyme for converting saturated into unsaturated fatty acids and is responsible for the introduction of the first double bond between carbon 9 and 10 of palmitoyl (16:0) or stearoyl (18:0)-CoA to form palmitoleic (16:1) or oleic (18:1) acid (18). In an attempt to elucidate the genetic basis of the difference in fatty acid composition (membrane fluidity) in different isolates of *H. capsulatum* and the mechanisms that regulate heat shock gene expression, we began a molecular analysis of the structure and transcriptional regulation of the gene coding for Δ^9-desaturase, a key enzyme responsible for unsaturated fatty acids formation.

Desaturases are responsible for regulating the appropriate level of desaturation in membrane lipids and are supposed to be key enzymes in modulating membrane fluidity (1,16). Δ^9-Desaturase genes have been cloned from *Saccharomyces cerevisiae* (19), rat (20), mouse (21,22), and humans (23). In this paper we report the cloning, the physical structure, and the transcriptional analysis of the Δ^9-desaturase gene from two strains of *H. capsulatum*. The two genes have been isolated from the genomic library of the virulent, temperature-tolerant G217B strain (normal level of heat shock gene expression) and from a library of the temperature-susceptible, avirulent Downs strain (reduced level of heat shock gene expression). We show that in the latter strain the Δ^9-desaturase gene is actively expressed in mycelia at 25°C while in the temperature-tolerant G217B strain no mRNA was detectable. The transcriptional data are in agreement with the analysis of fatty acids profile that showed the presence of a lower level of saturated fatty acids in mycelia of the Downs strain. Preliminary sequence analyses support the hypothesis that the Downs putative promoter is altered while the two coding regions are virtually identical. These data are discussed in relationship to the role of this enzyme in regulating membrane fluidity and in setting the threshold of the temperature at which heat shock transcription occurs.

MATERIALS AND METHODS

Organisms and preparation of cells for isolation of nucleic acids. Growth conditions and isolation of DNA from the temperature-susceptible avirulent Downs (ATCC38904; American Type Culture Collection, Rockville, MD) and the temperature-tolerant virulent G217B (ATCC26034) strains of *H. capsulatum* have been described previously (5). Total RNA was extracted from the mycelial phase of Downs grown at 25°C and from mycelia that had been incubated at a temperature of 34 or 37°C for 1, 3, 6, or 24 h after the temperature shift. Total RNA was also isolated from mycelia of strain G217B incubated at 25°C and from cells switched either 37 or 40°C for 1, 3, 6, and 24 h after the temperature shift. A 1.5-mL aliquot of the cells was centrifuged and resuspended in 470 µL of lysis buffer containing 100 mM sodium acetate pH 5.3 and 1 mM $MgCl_2$. Cell lysis was obtained in the presence of 5 µL of diethyl pyrocarbonate by adding 25 µL of 20% SDS. Nucleic acids were extracted with 1 vol of the mixture [(by vol phenol/chloroform/isoamyl alcohol (25:24:1)] preequilibrated at 65°C. The mixture was vortexed in the presence of approximately 200 µL of glass beads, allowed to settle, and then centrifuged. The aqueous phase was extracted three times with the above mixture at 65°C and once with $CHCl_3$/isoamyl alcohol (24:1) at room temperature. RNA was precipitated with 0.3 M sodium acetate, pH 5.3, and 3 vol of 95% ethanol at −20°C overnight.

Molecular cloning. The Δ^9-desaturase gene was cloned by polymerase chain reaction (PCR) amplification of genomic DNA of the G217B strain using two synthetic, degenerated oligo-deoxynucleotides corresponding to two conserved regions of the cloned genes (*S. cerevisiae* and rat). The two sequences 5′-ACNGCNGGNTAYCAYMGNYTNTGG-3′ and 5′-TTYTTNARRTCRTANGCNARNCC-3′ correspond to the amino acid sequences TAGYHRLW and GLAYDLKK. The amplified fragment of 651 nt was cloned in pUC18, sequenced, and used to screen two genomic libraries of G217B (λDASH) and Downs (λGT11) strains. The positive clones were analyzed, and the *Xba*I-containing gene fragments were subcloned in pUC18. Appropriate subclones were generated to sequence the entire genes and their flanking regions, using the dideoxynucleotide chain termination method (24). Both strands were sequenced. In some cases, appropriate oligo-deoxynucleotides were synthesized for sequencing particularly unclear regions. All the standard procedures and manipulations were according to Sambrook *et al.* (25). The enzymes were used according to the manufacturers' indications.

Transcription start site analysis by primer extension. An antisense oligonucleotide (5′-TCTGTGATATGAACCTTC-3′) was synthesized spanning from nucleotide +105 to +122 relative to the putative initiation translation site ATG. Five ng of the oligomer were 5′ end-labeled with [γ^{32}P]ATP (specific activity 3,000 Ci/mmole; Amersham Co., Chicago, IL) using 5 units of T4 polynucleotide kinase (BRL, Middlesex, United Kingdom). A mixture containing the oligomer and 10 or 30 µg of total RNA extracted from yeast cells grown at 37°C of strain G217 was denatured at 93°C for 2 min in avian myeloblastosis virus (AMV) reverse transcriptase buffer (0.5 M Tris-HCl, pH 8.3, 60 mM $MgCl_2$, 0.1 M DTT, 0.1 M NaCl) and then frozen in a dry ice/ethanol bath for 2 min. The solu-

tions were slowly thawed on ice. Primer extension reaction was carried out at 42°C for 30 min with 200 µM of a mixture containing the four cold nucleotides and 2 units of AMV reverse transcriptase (Boehringer, Mannheim, Germany). Samples were separated on a 6% polyacrylamide sequencing gel containing 7 M urea. After electrophoresis, gels were transferred to 3 MM Whatman paper, dried, and autoradiographed at −70°C.

Northern blot analysis. Fifteen µg of total RNA was precipitated in ethanol and resuspended in 1 × 3-(*N*-morpholino) propanesulfonic acid (MOPS) [1 × MOPS is 20 mM MOPS, 10 mM sodium acetate pH 5.3, and 1 mM EDTA], 50% formamide, 2.2 M formaldehyde, and heated 10 min at 65°C. Samples were separated by electrophoresis on 1% denaturing agarose gel in 1 × running buffer (1 × MOPS). RNA was then transferred onto nylon membranes in 20 × saline-sodium citrate (SSC) (Boehringer) and fixed by ultraviolet (UV) crosslinking. Filters were then hybridized using 10^7 cpm of [^{32}P]-probe DNA in 5 × SSC, 5 × Denhardt's, 50% formamide at 42°C overnight. The probe was labeled by multipriming to a specific activity of 10^9 cpm/µg, and filters were washed under high stringency conditions at 65°C for 40 min.

Total RNA was determined spectrophotometrically and by scanning an ethidium bromide gel containing the same amount of total RNA which were used for the Northern blot. The ethidium bromide gel was photographed, and rRNA bands were analyzed by the computer program Image 1.47 using a macro specifically designed to quantify gel bands. In addition, the filter, after hybridization, was stained 5 min in 5% acetic acid and 5 min in 0.04% methylene blue in 0.5 M sodium acetate followed by washing with water (not shown). The intensity of rRNA bands were further checked by the software program to make sure that the RNA loaded on the gel was uniformly transferred to filter. Filters that were hybridized with *cdc2* also have been hybridized with a fragment of *Hc* 18S rRNA gene to ensure that same amount of RNA was on filters (not shown). The exposed film was scanned and intensity of bands measured by the Image software (National Institutes of Health, Bethesda, MD).

RESULTS

Cloning of Δ9-fatty acid desaturase gene of thermotolerant G217B strain. Genomic DNA was amplified by PCR with two synthetic degenerated oligonucleotides corresponding to two conserved regions of cloned Δ^9-desaturase genes. The 651-nt fragment obtained by PCR amplification was first sequenced and then used as a probe to isolate the entire *Ole1* gene and its flanking sequences from a genomic λDASH (Stratagene Cloning systems, La Jolla, CA) library of strain G217B. About 10^5 recombinant phages were screened by hybridization with the PCR probe under stringent conditions. One of seven positive clones found was further subcloned in pUC18 vector and analyzed. The entire nucleotide sequence of G217B strain of *H. capsulatum Ole1* gene has been determined (GenBank accession number X85962, Heidelberg). Sequence analysis of the translated fragments revealed that the G217B *Ole1* gene contains a single open reading frame (ORF) starting from the first ATG of 1431-nt that is interrupted by one intron of 93 nt. The presence of the intron that, like other fungal introns, resembles those present in all eukaryotic mRNA having the acceptor and donor conserved consensus sequences following the GT/AG rule for 5′ and 3′ splice site (26), was confirmed by sequencing a cDNA clone with appropriate oligonucleotides on both sides of the intron.

Cloning of Δ^9-desaturase gene of thermosensitive Downs strain. Since strains of *H. capsulatum* exist exhibiting different temperatures of thermotolerance that correlate with optimum of heat shock gene transcription and level of virulence (5,8,10,12), we cloned the Δ^9-desaturase gene from a λGT11 genomic library of the thermosensitive Downs strain using as a probe the PCR product described above. One of the five positive phages were subcloned in pUC18 vector and analyzed (GenBank accession number X85963, Heidelberg), and one of them containing a 4 kb insert was sequenced. The coding sequence (476 aa) contained only seven amino acid substitutions compared to the coding sequence of *H. capsulatum* G217B strain.

The Downs strain contains an intron of 93 nt in the same location as that cloned from G217B. Both ORF code for a protein of approximately 53,700 Da. A screening against a representative protein sequence data base (PIRProtein, release 36) showed a significant similarity to Δ^9-desaturase proteins of *S. cerevisiae* (72% similarity, 49% aa identity), rat (55.3 and 31.5%, respectively), mouse (56 and 34%, respectively), and human (57 and 34%, respectively, human gene has only been partially cloned). Comparisons were done using the GCG sequence analysis program *Gap*. Long stretches of highly conserved amino acids are present, as between aa 270 and 298 of *H. capsulatum* and aa 330 and 358 of *S. cerevisiae* with 25 out 28 identical aa, demonstrating that our cloned sequences coincide with a Δ^9-desaturase gene (Fig. 1). The most conserved amino acid is histidine with 18 out 22 residues perfectly aligned (27,28). Arginine residues of the rat liver enzyme have been previously identified as being involved in substrate binding (29). It is interesting to note that arginine residues are also highly conserved in *H. capsulatum Ole1* genes. Furthermore, it has been shown that both the rat liver and the *S. cerevisiae* enzyme have striking similarities in hydropathic characteristics (27,28). Both proteins have been shown to contain two regions of 50 aa containing long stretches of hydrophobic residues forming loops that have been proposed to be responsible for the anchoring of the protein in the endoplasmic reticulum (27). These hydrophobic regions are in identical position in the aligned *S. cerevisiae* and rat proteins (27). Figure 1 shows that the *Ole1* predicted proteins of *H. capsulatum* contain similar stretches of amino acids located in the same position confirming the presence of transmembrane loop regions.

Transcription start point analysis by 5′ end extension. The location of the putative 5′ end of *Ole1* mRNA was mapped by primer extension experiments for the G217B strain. A 17-

```
                                                                                                  22
H.c. ole-1                                                                                MALNEAPTATPAAETAAGKAVT
S.c. ole-1      mptsgttieliddqfpkddsassgivdevdlteanilatglnkkaprivngfgslmgskemvsvefdkkgnekksnldrl
rat  ole-1                                                                        mpahmlqeisssyttttttiEpPsgnlqngrekmKkVp
mus  ole-1                                                                        mpahilqeisgaysatttitapPsggqqnggekfekss
hum  ole-1                                                                        ahmlqedisssyttttttitapPpgvlqnggdkletmp

                                                                                                 102
H.c. ole-1      TDTARRPNPEPK-KVHITDTPITLANWHKHVS-WLNVTLIIVIPIYGLVQA--YWVPLHLKTALWAVVYYFMTGLGITAG
S.c. ole-1      lekdnqekeEaKtKiHIseqPwTLnNWHqHln-WLNmvLvcgmPmiGwyfAlsgkVPLHLnvfLfsVfYYavgGvsITAG
rat  ole-1      lyleedirPEmrediHdpsyqdeegpppKleyvWrNiiLmallhv-GalygitlipsskvyTlLWgifYYlisaLGITAG
mus  ole-1      hhwgadvrPElKddiydptyqddegpppKleyvWrNiiLmallhl-GalygitlvpsckLyTcLfAylYYvisaLGITAG
hum  ole-1      lyleddirPdiKddiydptykdkegpspKveyvWrNiiLmslllhl-GalygitliptckfyTwLWgVfYYFvsaLGITAG

                                                                                                 182
H.c. ole-1      YHRLWAHCSYSATLPLKIYLAAVGGGAVEGSIRWWARGHRAHHRYTDTDKDPYSAHKGLLYSHIGWMVMKQNPKRIGRTD
S.c. ole-1      YHRLWsHrSYSAhwPLrIfyAifGcasVEGSakWWghsHRiHHRYTDTlrDPYdArrGLwYSHmGWMllKpNPKykaRaD
rat  ole-1      aHRLWsHrtYkArLPLrIfLiiantmAfqndvyeWARdHRAHHkfseThaDPhnsrrGfffSHvGWllvrkhPavkekgg
mus  ole-1      aHRLWsHRtYkArLPLrlfLiiantmAfqndvyeWARdHRAHHkfseThaDPhnsrrGfffSHvGWllvrkhPavkekgg
hum  ole-1      aHRLWsHrSYkArLPLrlfLiiantmAfqndvyeWARdHRAHHkfseThaDPhnsrrGfffSHvGWllvrkhPavkekgs

                                                                                                 262
H.c. ole-1      ITDLNEDPV---VVWQHRNYLKVVIFMGIVFPMLVSGLGWGDWLGGFIYAGI-LRIFFVQQATFCVNSLAHWLGDQPFDD
S.c. ole-1      ITDmtdDwtirf---QHRhYillmlltafViPtLicGyffnDymGGlIYAGf-iRvFviQQATFCiNSmAHyiGtQPFDD
rat  ole-1      klDmsdlkaeklVmfQrRyYkpglllMcfilPtLVpwycWGetflhslfvstfLRytlVlnATwlVNSaAHlyGyrPyDk
mus  ole-1      klDmsdlkaeklVmfQrRyYkpdlllMcfVlPtLVpwycWGetfvnslcvstfLRyavVlnATwlVNSaAHlyGyrPyDk
hum  ole-1      tlDLsdleaeklVmfQrRyYkpgllmMcfilPtLVpwycWG

                                                                                                 342
H.c. ole-1      RNSPRDHIVTALVTLGEGYHNFHHEFPSDYRNAIE-WHQYDPTKWSIWIWKQLGLAYDLKQFRANEIEKGRVQQLQKKID
S.c. ole-1      RrtPRDnwiTAiVTFGEGYHNFHHEFPtDYRNAIK-WyQYDPTKviIyltslvGLAYDLKkFsqNaIEealiQQeQKKIn
rat  ole-1      niqsRenIlvsLgsvGEGfHNyHHaFPyDYsaseyrWHinftTff-IdcmaaLGLAYDrKkvskaavlarikrtgdgshk
mus  ole-1      niSsRenIlvsmgavGErfHNyHHaFPyDYsaseyrWHinftTff-IdcmalLGLAYDrKrvsraavlarikrtgdgsck

                                                                                                 422
H.c. ole-1      QRRVKLDWGIPLDQLPVIEWDDYVDQAKNGRGLIAIAGVVHDVTDFIKDHPGGKAMINTGIGKDATAMFNGGVYNHSNAA
S.c. ole-1      kkkaKinWGpvLtdLPmwdkqtflaksKenkGLviIsGiVHDVsgyIseHPGGetlIkTalGKDATkaFsGGcYrHSNAA
rat  ole-1      ss
mus  ole-1      sg

H.c. ole-1      HNLLSTMRVGVIRGGCEVEIWKRAQKEKKEVESVRDEYGNRIVRAGAQVTKIPEPITTADAA
S.c. ole-1      qNvLadMRVaVIkesknsairmaskrgeiiyetgkff
```

FIG. 1. Comparative analysis of the amino acid sequences of Δ^9-desaturase gene products of *Histoplasma capsulatum* (*H.C.*), *Saccharomyces cerevisiae* (*S.C.*), rat, mouse (mus), and humans (hum). Boxes indicate the highly conserved histidine residues. Lower-case letters indicate residues that are not identical to those in the *H.C.* protein.

nt antisense oligodeoxynucleotide (5′-TCTGTGATATGAACCTTC-3′) was synthesized spanning from nt +105 to +122 relative to the putative start codon ATG and annealed to total RNA extracted from yeast cells grown at 37°C. The result in Figure 2 demonstrates the presence of one major extended molecule of 253 nt. This experiment defines an untranslated 5′ region of 131 nt from the putative translation start codon. Previously, we also found two untranslated regions of 163 and 186 nt in the *hsp82* gene (7) and one of 218 nt in the *cdc2* gene of *H. capsulatum* (30). Search of TATA-like consensus sequence upstream of the transcription start point did not identify any consensus in both sequences.

Analysis of Ole1 expression. We previously showed that the two strains of *H. capsulatum* contain differences in the membrane saturated/unsaturated fatty acid ratio (Carratù, L., Franceschelli, S., Pardini, C.L., Kobayashi, G.S., Horvath, I., Vigh, L., and Maresca, B., manuscript in preparation). Assuming there are no differences in the coding sequences of the two cloned *Ole1* genes, we have investigated transcription of *Ole1* gene in both strains. Northern experiments were performed using total RNA extracted from cells growing at 25°C and then shifted up to 34, 37, and 40°C and from these temperatures to 25°C. Figure 3 shows hybridization of total RNA extracted from G217B mycelia (Fig. 3A) at various times after the shift from 25 to 37°C and from 37 to 25°C. *Ole1* gene expression is poorly detectable in cells growing at 25°C and, when shifted at 37°C, expression can be measurable 6 h from temperature shift. In yeast cells growing at 37°C, the gene is actively transcribed at this temperature, and as expected (31,32), expression was enhanced after shift down to 25°C. When the mycelium-to-yeast phase transition was accomplished at the upper temperature range for this strain (40°C), a similar pattern of expression was observed (Fig. 3B). However, cells required more than 24 h to restore transcription at 40°C, and, after the shift to 25°C, the gene was more rapidly switched off. On the other hand, while transcription of *Ole1* gene in the thermosensitive Downs strain is similar to that of G217B, a striking difference was measurable in mycelia grown at 25°C. Figure 3C shows that *Ole1* gene is actively transcribed at 25°C and switched off when

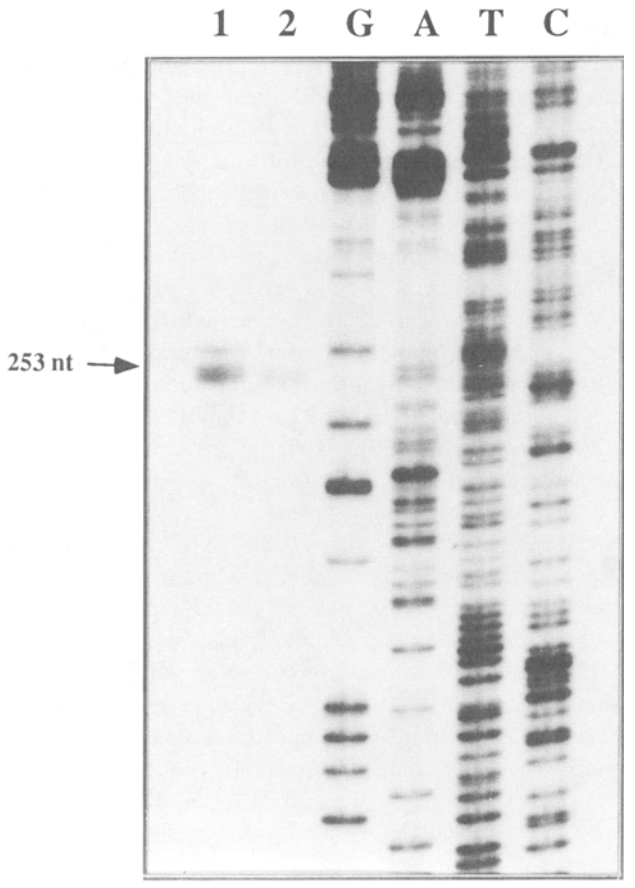


FIG. 2. Location of the 5′ end of *Histoplasma capsulatum* Δ^9-desaturase mRNA by primer extension. An antisense oligonucleotide (5′-TCTGT-GATATGAACCTTC-3′) was synthesized spanning from nucleotide +105 to +122 relative to the putative initiation translation site ATG. Five ng of the oligomer were 5′ end-labeled with [γ^{32}P]ATP. Lane 1: 30 µg total RNA; Lane 2: 15 µg total RNA. Lanes marked G to C are the sequence ladder of DNA of known sequence. Samples were separated on a 6% polyacrylamide sequencing gel containing 7 M urea.

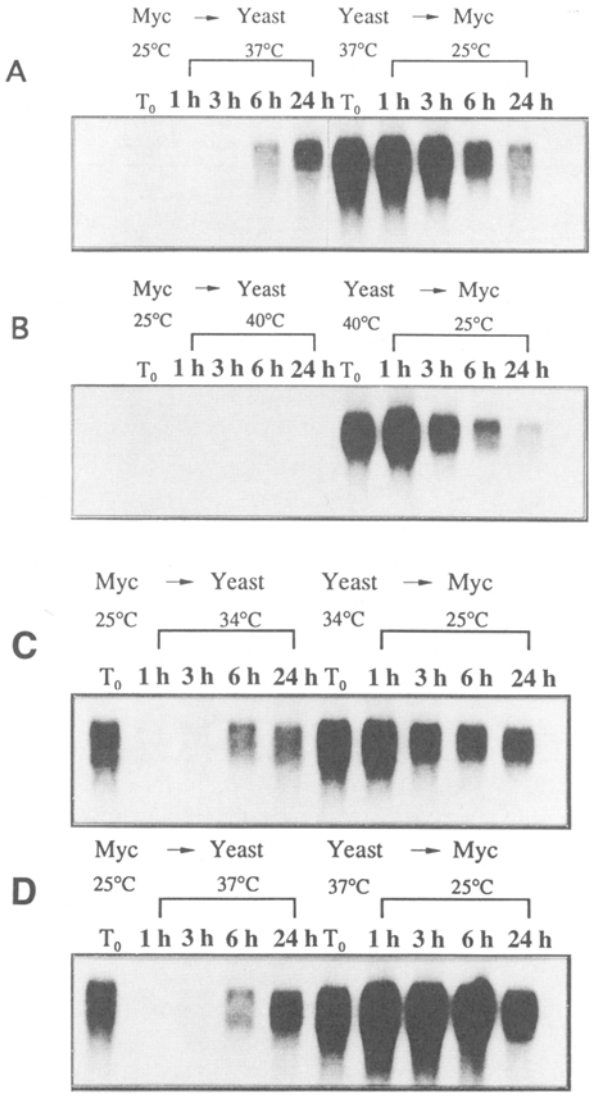


FIG. 3. Northern blot of total RNA purified of *Histoplasma capsulatum* Δ^9-desaturase gene in G217B and Downs. A: Shows hybridization of RNA purified from mycelia (Myc) cells grown at 25°C and from mycelia 1, 3, 6, and 24 h after the shift to 37°C and from yeast grown at 37°C and 1, 3, 6, and 24 h after shift to 25°C. B: Shows an identical experiment, but 40°C was used rather than 37°C. C: Shows hybridization of RNA purified from mycelia cells grown at 25°C and from mycelia 1, 3, 6, and 24 h after the shift to 34°C and from yeast grown at 34°C and 1, 3, 6, and 24 h after shift to 25°C. D: Shows an identical experiment, but 37°C was used rather than 34°C. Fifteen µg of total RNA were loaded for each sample.

temperature was raised either to 34 (Fig. 3C) or 37°C (Fig. 3D), the latter being the upper temperature range for this strain (8). Figure 4 shows a time-course experiment of the effect of temperature shift 25 to 37°C in both strains showing that *Ole1* gene is turned off within 30 min from temperature shift up. These experiments clearly indicate that the *Ole1* gene transcription is altered in the Downs strain.

Thus far, we have sequenced about 900 nt upstream to the ATG of both *Ole1* sequences (data not shown). A preliminary analysis of these sequences shows that while a high conservation in this region has been retained in these genes, major differences have been found in a specific region (data not shown). Though no conclusions can be drawn from these observations at the present time, it is reasonable to speculate that the difference in transcriptional regulation between the two *Ole1* genes may lie in this region of low nucleotide identity.

DISCUSSION

Recently, we demonstrated that, in the two strains of *H. capsulatum* that differ in their optimal temperature of heat shock mRNA induction, it is possible to modulate transcription of these genes by exogenous addition of fatty acids (14). In the temperature-susceptible Downs strain exposure of mycelia to saturated fatty acids at 25°C induced a strong increase in heat shock gene transcription when cells were shifted to 37°C. On

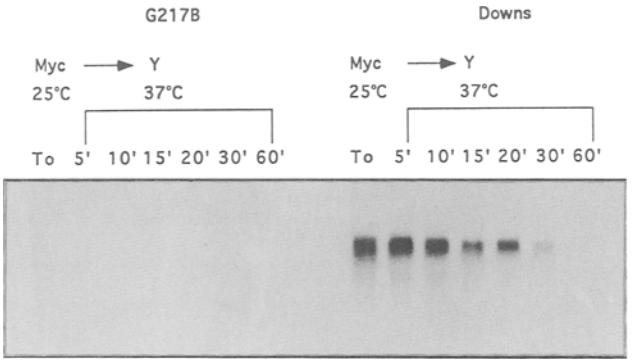


FIG. 4. Time course of the expression of of *Histoplasma capsulatum* Δ^9-desaturase gene in G217B and Downs strains after a temperature shift from 25 to 37°C.

the other hand, in the temperature-tolerant G217B strain addition of unsaturated fatty acids to mycelia drastically reduced heat shock gene transcription when cells were shifted to 37°C. We further observed that the Downs strain has a higher unsaturated fatty acid content, particularly oleic acid; on the other hand, the membrane of the G217B strain has a lower unsaturated fatty acids content and is less fluid (Carratù, L., Franceschelli, S., Pardini, C.L., Kobayashi, G.S., Horvath, I., Vigh, L., and Maresca, B., manuscript in preparation). Thus, these studies suggest that the level of saturation of membrane fatty acids may be involved in the perception of sudden temperature changes (heat shock).

We cloned the Δ^9-desaturase gene by PCR amplification of genomic DNA using two synthetic, degenerated oligo-deoxynucleotides corresponding to two conserved regions of the cloned genes from both the temperature-sensitive Downs and from the temperature-tolerant G217B strains. The 651 nt amplified fragment was cloned in pUC18, and, after sequencing to confirm that it corresponded to a Δ^9-fatty acid desaturase gene, was used to screen two genomic libraries of G217B (λDASH) and Downs (λGT11) strains. The positive clones were analyzed, and the *Xba*I-containing gene fragments were subcloned in pUC18. Appropriate subclones were generated to sequence the entire genes and their flanking regions. In contrast to the *S. cerevisiae Ole1* gene that lacks introns (27), the *H. capsulatum Ole1* gene of both strains contains an ORF starting from the first ATG of a single 1431-nt ORF interrupted by one intron, of 93 nt. Like other fungal introns, the 93 nt sequence resembles those present in all eukaryotic mRNA having the acceptor and donor conserved consensus sequences following the GT/AG rule for 5′ and 3′ splice site (26), which was confirmed by sequencing a cDNA clone with appropriate oligonucleotides on both sides of the intron. Screening against a representative protein sequence data base showed a good degree of homology to *S. cerevisiae* (476 aa, in contrast to 510 aa for the *Ole1* protein of *S. cerevisiae*). The two coding sequences cloned from both strains of *H. capsulatum* are virtually identical with only seven amino acid differences between the two sequences.

Both ORF code for a protein of approximately 53,700 Da (57,400 in *S. cerevisiae*). A screening against a representative protein sequence data base (PIRProtein), using the GCG sequence analysis program *Gap*, showed a significant similarity to *S. cerevisiae* (72% similarity, 49% aa identity), rat (55.3 and 31.5%, respectively), mouse (56 and 34%, respectively), and human (57 and 34%, respectively). The human gene, however, has only been partially cloned (23). Long stretches of highly conserved amino acids, as between aa 270 and 298 of *H. capsulatum* and aa 330 and 358 of *S. cerevisiae* with 25 out 28 identical aa, were identified, demonstrating that our cloned sequences coincide with a Δ^9-desaturase gene. More recently it has been shown that the most conserved amino acid is histidine with 18 out of 22 residues perfectly aligned (28). Furthermore, 8 histidine residues are essential in membrane-associated stearoyl-CoA desaturases, and the relative positions of these residues are conserved in alkane hydroxylases and xylane monoxygenases (28). All of the 8 most conserved His residues that have been shown by mutations (His→Ala) in the rat Δ^9-desaturase gene to be necessary for growth in a minimal medium without unsaturated fatty acids (28) are present in the *Histoplasma Ole1* gene. These His residues have been suggested to have an essential catalytic function. These authors have identified three regions of primary sequence containing conserved His residues. These sequences defined as HX_4H, HX_2HH, and HX_2HH are all present in both *H. capsulatum* Δ^9-desaturase proteins. Though the importance of His in these sequences has not been elucidated, it is interesting to note that a consensus containing these three sets of sequences is present only in 21 of all proteins sequenced, and 18 are fatty acid desaturases, including the Δ^9-desaturase of *H. capsulatum*.

Arginine residues of the rat liver enzyme have been previously identified as being involved in substrate binding (29). We also found that arginine residues in the *H. capsulatum Ole1* genes are highly conserved. Furthermore, previously it has been shown that both the rat liver and the *S. cerevisiae* enzyme have striking similarities in hydropathic characteristics (27). Both proteins contain two regions of 50 aa containing long stretches of hydrophobic residues forming loops that have been proposed to be responsible for the anchoring of the protein in the endoplasmic reticulum (27). These hydrophobic regions are in identical position in the aligned *S. cerevisiae* and rat proteins (27). Figure 1 shows that the *H. capsulatum Ole1* predicted proteins containing such stretches are located in the same position, and confirm the presence of such transmembrane loop regions.

We have determined the transcriptional start site of *Ole1* gene by primer extension demonstrating the presence of one major extended molecule defining an untranslated 5′ region of 131 nt from the translation start codon. Similarly, short 5′ untranslated regions were detected in *hsp82* (5) and *cdc2* (30) genes of *H. capsulatum*.

The *Ole1* gene was transcribed in mycelia of Downs strain grown at 25°C while a shift to 37°C caused an abrupt cessation of expression which lasted at least 3 h and resumed 24 h

after the temperature shift. On the contrary, when mRNA purified from G217B strain was probed, no transcription was detectable in mycelia at 25°C nor in the first 3 h of the temperature shift to 37°C. When yeast cells growing at 37°C were incubated at 25°C, there was a burst in transcription 1 h after temperatures shift down and then transcription resumed at normal rate both in Downs and G217B strain. When a time course experiment was performed in Downs mycelia shifted from 25 to 37°C, it was shown that within 30 min after temperature change *Ole1* gene transcription was turned off (Fig. 4). These transcriptional analyses are in agreement with the differences in saturated fatty acids we found in the two strains. In fact, the lower amount of saturated fatty acids measured in the Downs strain can be explained by the differences in transcriptional regulation of the *Ole1* gene.

It is interesting to note that the *Ole1* gene of *H. capsulatum* is highly expressed in yeast cells of both Downs (at 34 and 37°C) and G217B (37 and 40°C) strains before the shift down to 25°C. The burst in transcription lasted up to 6 h in both strains to decrease but was still present after 24 h of the shift down (yeast-to-mycelium morphologic transition). However, the amount of mRNA present in Downs strain was much higher (Fig. 3). Cells fully transform in 3 to 4 d in the case of G217B while 6–7 d are required for the more temperature-sensitive Downs (8). In the case of the G217B strain, fully transformed cells growing as mycelia at 25°C do not express *Ole1* while Downs cells maintain a high level of expression. We also detected *Ole1* mRNA transcription in mycelia grown at 25°C and then switched to 34 or 37°C in the case of Downs and to 37°C in the case of G217B between 6 and 24 h after temperature shift up. To our knowledge, this is first evidence describing transcriptional induction of a Δ^9-desaturase gene as a result of temperature increase, though it occurs after 6 h after the temperature shift. Nevertheless, we can not exclude that the induction of the desaturase is a result of morphogenesis rather than a consequence of the temperature change. *Histoplasma capsulatum* is a dimorphic fungus, and as other parasites, it adapts to the new conditions present in the host, such as higher temperature, changes in redox potential and pH, different nutrients, etc. It is reasonable to postulate that, among other changes, also a different membrane lipid composition is part of the adaptive process that leads to the morphologic mycelium-to-yeast transition. In addition, morphogenesis comprises a heat shock phenomenon (rapid temperature shift up from 25 to 37°C) that involves induction of a specific set of genes (heat shock genes) that in cohort with others permit adaptation to the new temperature. Our data suggest that the Δ^9-desaturase gene may be involved in the adaptation to a higher temperature during morphogenesis and in the process of reorganization of the membrane of the yeast form.

In summary, we have shown that the Downs strain of *H. capsulatum*, which is altered in its saturated/unsaturated fatty acids ratio, nonvirulent and temperature-susceptible has a different optimal temperature of expression of heat shock gene. Expression studies of cloned *hsp70* (6) and *hsp82* (5) genes revealed that 34°C is the temperature of maximal heat shock mRNA transcription in this strain, while in virulent strains, such as G217B, maximal induction of heat shock gene expression occurs at 37°C. Our results indicate that the cell membrane is a primary target in the biological perception of temperature during rapid temperature changes and propose that saturated/unsaturated fatty acids ratio and lipoprotein membrane complexes are involved in the perception of temperature changes and that under heat shock conditions disturbance of the physical state of the membrane causes transduction of a signal that induces transcription of heat shock genes.

ACKNOWLEDGMENTS

This work was supported by a Contract from Ministero della Sanità - Istituto Superiore di Sanità, VIII Progetto AIDS 1995 and NIH grant AI 29609.

REFERENCES

1. Hazel, J.R., and Williams, E.E. (1990) The Role of Alterations in Membrane Lipid Composition in Enabling Physiological Adaptation of Organisms to Their Physical Environment, *Progr. Lipid Res. 29*, 167–227.
2. Cossins, A.R., and Raynard, R.S. (1987) Adaptive Response of Animal Cell Membrane to Temperature, *Symp. Soc. Exp. Biol. 41*, 95–111.
3. Cossins, A.R., and Prosser, C.L. (1978) Evolutionary Adaptations of Membranes to Temperature, *Proc. Natl. Acad. Sci. USA 75*, 2040–2043.
4. Lindquist, S. (1986) The Heat Shock Response, *Ann. Rev. Biochem. 55*, 1151–1191.
5. Minchiotti, G., Gargano, S., and Maresca, B. (1991) The Intron-containing *hsp82* Gene of the Dimorphic Pathogenic Fungus *Histoplasma capsulatum* Is Properly Spliced in Severe Heat Shock Conditions, *Mol. Cell. Biol. 11*, 5624–5630.
6. Caruso, M., Sacco, M., Medoff, G., and Maresca, B. (1987) Heat Shock 70 Gene Is Differentially Expressed in *Histoplasma capsulatum* Strains with Different Levels of Thermotolerance and Pathogenicity, *Mol. Microbiol. 1*, 151–158.
7. Ajello, L. (1971) Coccidioidomycosis and Histoplasmosis: A Review of Their Epidemiology and Geographic Distribution, *Mycopathol. Mycol. Appl. 45*, 221–230.
8. Maresca, B., and Kobayashi, G.S. (1989) Dimorphism in *Histoplasma capsulatum*: A Model for the Study of Cell Differentiation in Pathogenic Fungi, *Microbiol. Rev. 53*, 186–209.
9. Medoff, G., Sacco, M., Maresca, B., Schlessinger, D., Painter, A., Kobayashi, G.S. and Carratù, L. (1986) Irriversible Block of the Mycelial to Yeast Phase Transition of *H. Capsulatum. Science 231*, 476–479.
10. Maresca, B., Carratù, L., and Kobayashi, G.S. (1994) Morphologic Transition in the Human Fungal Pathogen *Histoplasma capsulatum, Trends Microbiol. 2*, 110–114.
11. Kobayashi, G.S. and Maresca, B. (1993) Dimorphism in *Histoplasma capsulatum:* Study of Cell Differentiation and Adaptation, in *Dimorphic Fungi in Biology and Medicine* (van den Bossche, H., Odds, F., and Kerridge, D., eds.) pp. 213–218, Plenum Press, New York.
12. Medoff, G., Maresca, B., Lambowitz, A.M., Kobayashi, G.S., Painter, A., Sacco, M., and Carratù, L. (1986) Correlation Between Pathogenicity and Temperature Sensitivity in Different Strains of *Histoplasma capsulatum, J. Clin. Inv. 78*, 1638–1647.
13. Maresca, B., and Carratù, L. (1992) The Biology of the Heat Shock Response in Parasites, *Parasitol. Today 8*, 260–266.

14. Maresca, B., and Kobayashi, G.S. (1993) Changes in Membrane Fluidity Modulate Heat Shock Gene Expression and Produce Attenuated Strains in the Dimorphic Fungus *Histoplasma capsulatum, Arch. Med. Res. 24*, 247–249.
15. Maresca, B., and Cossins, A. (1993) Fatty Feedback and Fluidity. News and Views, *Nature 365*, 606–607.
16. Macartney, A., Maresca, B., and Cossins, A.R. (1994) Acyl-CoA Desaturases and the Adaptative Regulation of Membrane Lipid Composition, in *Temperature Adaptation of Biological Membranes* (Cossins, A., ed.) pp. 129–139, Portland Press, London.
17. Broomfield, D.K., and Block, K. (1960) The Formation of Δ^9-Unsaturated Fatty Acids, *J. Biol. Chem. 235*, 337–344.
18. Strittmatter, P.L., Spatz, L., Corcoran, D., Rogers, M.J., Setlow, B., and Redline, R. (1974) Purification and Properties of Rat Liver Microsomal Stearoyl CoA Desaturase, *Proc. Natl. Acad. Sci. USA 71*, 4565–4569.
19. Stukey, J.E., McDonough, V.M., and Martin, C.E. (1989) Isolation and Characterization of OLE1, a Gene Affecting Fatty Acid Desaturation from *Saccharomyces cerevisiae*, *J. Biol. Chem. 264*, 16537–16544.
20. Mihara, K. (1990) Structure and Regulation of Rat Liver Microsomal Stearoyl-CoA Desaturase Gene, *J. Biochem. (Tokyo) 108*, 1022–1029.
21. Ntambi, J.M., Buhrow, S.A., Kaestner, K.H., Christy, R.J., Sibley, E., Kelly, T.J.J., and Lane, M.D. (1988) Differentiation-Induced Gene Expression in 3T3-L1 Preadipocytes. Characterization of a Differentially Expressed Gene Encoding Stearoyl-CoA Desaturase, *J. Biol. Chem. 263*, 17291–17300.
22. Kaestner, K.H., Ntambi, J.M., Kelly, T.J.J., and Lane, M.D. (1989) Differentiation-Induced Gene Expression in 3T3-L1 Preadipocytes. A Second Differentially Expressed Gene Encoding Stearoyl-CoA Desaturase, *J. Biol. Chem. 264*, 14755–14761.
23. Li, J., Ding, S.-F., Habib, N.A., Fermor, B.F., Wood, C.B., and Gilmour, R.S. (1994) Partial Characterization of a cDNA for Human Stearoyl-CoA Desaturase and Changes in Its mRNA Expression in Some Normal and Malignant Tissues, *Int. J. Cancer 57*, 348–352.
24. Hattori, M., and Sakaki, Y. (1986) Dideoxy Sequencing Method Using Denatured Plasmid Templates, *Anal. Biochem. 152*, 232–238.
25. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2nd edn., Cold Spring Harbor.
26. Mount, S.M. (1982) A Catalog of Splice Junction Sequences, *Nucleic Acids Res. 10*, 459–472.
27. Stukey, J.E., McDonough, V.M., and Martin, C.E. (1990) The OLE1 Gene of *Saccharomyces cerevisiae* Encodes the Delta 9 Fatty Acid Desaturase and Can Be Functionally Replaced by the Rat Stearoyl-CoA Desaturase Gene, *J. Biol. Chem. 265*, 20144–20149.
28. Shanklin, J., Whittle, E., and Fox, B.G. (1994) Eight Histidine Residues Are Catalytically Essential in a Membrane-Associated Iron Enzyme, Stearoyl-CoA Desaturase, and Are Conserved in Alkane Hydroxylase and Xylane Monooxygenase, *Biochemistry 33*, 12787–12794.
29. Enoch, H.G., and Strittmatter, P. (1978) Role of Tyrosyl and Arginyl Residues in Rat Liver Microsomal Stearylcoenzyme A Desaturase, *Biochemistry 17*, 4927–4932.
30. Di Lallo, G., Gargano, S., and Maresca, B. (1994) The *Histoplasma capsulatum cdc2* Gene Is Transcriptionally Regulated During the Morphologic Transition, *Gene 140*, 51–57.
31. Wodtke, E., and Cossins, A.R. (1991) Rapid Cold-Induced Changes of Membrane Order and Delta 9-Desaturase Activity in Endoplasmic Reticulum of Carp Liver: A Time-Course Study of Thermal Acclimation, *Biochim. Biophys. Acta. 1064*, 343–350.
32. Los, D., Horvath, I., Vigh, L., and Murata, N. (1993) The Temperature-Dependent Expression of the Desaturase Gene desA in *Synechocystis* PCC6803, *FEBS Lett. 318*, 57–60.

[Received April 10, 1995; in final revised form August 2, 1995; Revision accepted August 14, 1995]

Intestinal and Liver Fatty Acid Binding Proteins Differentially Affect Fatty Acid Uptake and Esterification in L-Cells

Daniel R. Prows[1], Eric J. Murphy and Friedhelm Schroeder*

Department of Physiology and Pharmacology, Texas A&M University, College Station, Texas 77843-4466

ABSTRACT: Differential effects of intestinal (I-FABP) or liver (L-FABP) fatty acid binding proteins on fatty acid uptake and esterification were examined using transfected mouse L-cell fibroblasts. L-FABP, but not I-FABP, expression increased the initial rate and extent of *cis*-parinaric acid uptake by 50 and 29%, respectively, compared to control cells. I-FABP and L-FABP expression preferentially increased [^{3}H]-oleic acid incorporation into triacylglycerols by 5.5-fold and 3.8-fold, respectively. While both L-FABP and I-FABP increased esterification of [^{3}H]-oleic acid into ethanolamine glycerophospholipids, these proteins had opposite effect on esterification into choline glycerophospholipids. These data show for the first time that distinct FABP differentially affect both fatty acid uptake and intracellular esterification.

Lipids 30, 907–910 (1995).

Intestinal fatty acid binding protein (I-FABP) and liver fatty acid binding protein (L-FABP) are homologous proteins produced by intestinal absorptive columnar epithelial cells (enterocytes). L-FABP is also expressed at high levels in hepatocytes (1,2). I-FABP is a 131-residue, 15.1 kDa, cytosolic protein that noncovalently binds one mole of long-chain (C_{16}–C_{20}) saturated or unsaturated fatty acid with K_d values between 1–4 μM (3,4). L-FABP is a 127-residue, 14.3 kDa, cytosolic protein that binds two moles of saturated or unsaturated fatty acid or one mole each of fatty acid and sterol (3,5). The K_d values for saturated and unsaturated fatty acids are between 1.4–2.6 μM (3). Studies *in vitro* show that FABPs stimulate fatty acid uptake, act as intracellular fatty acid carriers, and affect lipid synthetic enzyme activities (3,5–8). FABPs binds numerous hydrophobic ligands including cholesterol (5), hydroperoxy- and hydroxy-arachidonic acid metabolites (9), heme (10), prostaglandin E_1 (11), and acyl-CoA (8). However, the physiological function of FABPs in intact cells is not known. Experiments presented here utilized an identical transfected cell model system (L-cells) expressing either I-FABP or L-FABP. Parallel studies using separate cell lines give insight into their plausible distinct roles in intracellular fatty acid trafficking and esterification.

MATERIALS AND METHODS

Cells. Mouse L-cell fibroblasts (aprt$^-$, tk$^-$) expressing L-FABP were obtained as stated earlier (12). The aprt$^-$, tk$^-$ expressing I-FABP were prepared in the same manner. Briefly, cells were transfected with the cDNA for rat I-FABP by the calcium phosphate-DNA precipitation method (13), using the modification of Wigler *et al.* (14). Expression of I-FABP or L-FABP was determined by quantitative Western blot immunoassay using the respective antisera (12). High expression clones, in which the respective proteins represented 0.35–0.4% cytosolic proteins, were isolated and compared to control and mock-transfected control L-cells.

Cis-*parinaric acid assay. Cis*-parinaric acid (9,11,13,15 *cis, trans, trans, cis*-octadecatetraenoic acid; (Molecular Probes, Eugene, OR) in aqueous buffer is nonfluorescent, but fluorescence intensity increases upon association with a hydrophobic environment. Both L-FABP and I-FABP bind *cis*-parinaric acid very well, as measured by increased fluorescence intensity (3). Fluorescent *cis*-parinaric acid uptake studies were performed on cell suspensions at a final concentration of 1.5×10^5 cells/mL phosphate buffered saline in a total volume of 2 mL. Cells were continuously stirred at 37°C with a Bel-Art cell spinbar (Fisher Scientific, Pittsburgh, PA) in the sample compartment of a photon counting fluorimeter (ISS Instruments, Champaign, IL). *Cis*-parinaric acid (1.8 μM) was added with a Hamilton syringe (Reno, NV).

[^{3}*H*]*-oleic acid incorporation assay.* Cells were washed with phosphate buffered saline and incubated for 30 min at 37°C with serum-free medium containing [^{3}H]-oleic acid diluted with cold oleic acid to a final specific activity of 1 μCi/nmol and a concentration of 1.8 μM. Cellular lipids were extracted, resolved, and quantitated as described earlier (15–17).

Statistics. Statistical analysis was done using one-way analysis of variance combined with the Tukey-Kramer multiple comparisons test (InStat, GraphPad Software, San Diego, CA) or the Student's *t*-test.

[1]Present Address: Department of Environmental Health and Physiology/Biophysics, University of Cincinnati Medical Center, Cincinnati, OH 45267-182.

*To whom correspondence should be addressed at Department of Physiology and Pharmacology, Texas A&M University, TVMC College Station, Texas 77843-4466.

Abbreviations: I-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein.

Copyright © 1995 by AOCS Press

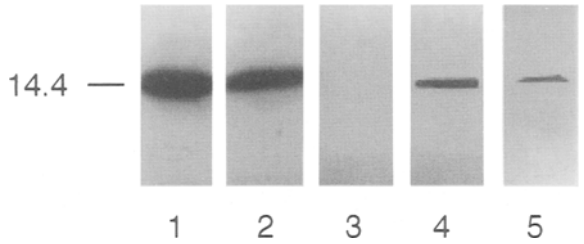


FIG. 1. Western blot showing intestinal fatty acid (I-FABP) and liver fatty acid protein (L-FABP) expression in transfected L-cell fibroblast. Lane 1 is I-FABP standard, and lane 2 shows I-FABP expression in total cell homogenate from transfected I-FABP expressing cells. Lane 3 is representative of total cell homogenate from nontransfected L-cells reacted with α-I-FABP. Lane 4 is L-FABP standard, and lane 5 shows L-FABP expression in total cell homogenate from transfected L-FABP expressing cells.

TABLE 1
***Cis*-Parinaric Acid Uptake Rates and Maximal Fluorescence**

Cell line	Initial rates (fluorescence units/s)	Maximal fluorescence (fluorescence units)
Control cells	220 ± 7.0 (12)	5534 ± 91 (12)
Mock-transfected cells	225 ± 8.7 (10)	5763 ± 82 (10)
High-I-FABP cells	222 ± 7.6 (18)	5897 ± 184 (17)
High L-FABP cells	337 ± 4.7[b] (6)	7128 ± 89[b] (6)

[a]*Cis*-parinaric acid uptake in control cells, mock-transfected control cells, high intestinal fatty acid binding proteins (I-FABP), and high liver fatty acid binding protein (L-FABP) expressing cells was determined as described in the Materials and Methods section. Initial rates are reported as the slope of the uptake curves (fluorescence vs. time) for the initial 10 s after fatty acid addition and mixing. Maximal fluorescence, which represents extent of total uptake, is given in relative fluorescence. Values are the mean ± SEM, with n = 6–18.

[b]$P < 0.001$ from all other cell lines.

RESULTS

Both I-FABP and L-FABP expressing cells had measurable cytosolic levels of their respective proteins (Fig. 1). L-FABP accounted for 0.40% of the total cytosolic protein in L-FABP expressing cells. I-FABP accounted for 0.35% of the total cytosolic protein in I-FABP expressing cells.

Cis-parinaric acid uptake into control, I-FABP expressing, and L-FABP expressing cells was linear for approximately the first 30 s and reached a plateau after approximately 1 min that was maintained for the duration of the assay (Fig. 2). *Cis*-parinaric acid uptake rates were significantly ($P < 0.001$) increased by 50% in L-FABP expressing cells compared to control or I-FABP expressing cells (Table 1). Maximal fluorescence indicates the extent of *cis*-parinaric acid uptake. L-FABP significantly ($P < 0.001$) increased the extent of *cis*-parinaric acid uptake by 29 and 21% compared to control and I-FABP expressing cells, respectively (Table 1). No significant differences in *cis*-parinaric acid uptake or maximal fluorescence were found between the two control cell lines or the high I-FABP expressing clones.

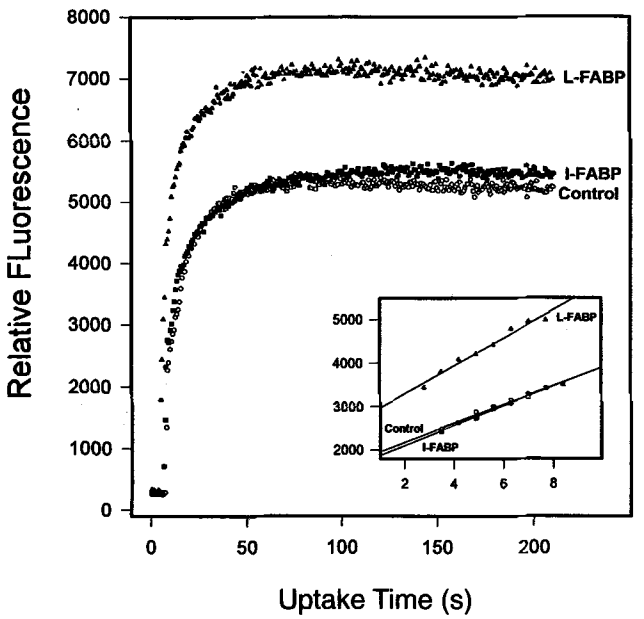


FIG. 2. *Cis*-parinaric acid uptake vs. time curves. *Cis*-parinaric acid (1.8 μM) was added to a continuously stirred cell suspensions (2 mL) in quartz cuvettes at 37°C. Increase in fluorescence over time represents binding and internalization of the fatty acid. Each curve is a representative assay with n = 6–18. Differences (SEM) between runs within the same cell line were always <10%. Inset represents initial readings following *cis*-parinaric acid addition. Initial uptake rates for the various cell lines were determined from the slope of the regression line for the initial readings. See Figure 1 for abbreviations.

Following [^{3}H]-oleic acid incorporation for 30 min, the esterified neutral and phospholipids were separated by high-performance liquid chromatography into individual lipid classes and the radiolabel distribution was determined (Table 2). No significant differences for any lipid classes were observed between the L-cells and the mock-transfected cells. These two groups were subsequently combined and referred to as control cells. No significant differences in nonesterified fatty acid proportions were found between any cell lines tested. In high I-FABP expressing cells, cholesteryl esters, triacylglycerols, and ethanolamine glycerophospholipids had significantly more [^{3}H]-oleic acid incorporated than control cells. However, the distribution of [^{3}H]-oleic acid into choline glycerophospholipids was significantly decreased in high I-FABP expressing cells compared to control cells. For high L-FABP expressing cells, [^{3}H]-oleic acid distribution into triacylglycerols, ethanolamine- and choline glycerophospholipids was significantly increased compared to control cells, while [^{3}H]-oleic acid distribution into cholesteryl esters was decreased. The total [^{3}H]-oleic acid incorporation was not significantly different between high I-FABP expressing cells and control cells; however, the total [^{3}H]-oleic acid incorporation by the L-FABP expressing cells was increased by 45% over control cells (data not shown).

Cis-parinaric acid was only slightly esterified in L-FABP expressing cells over a 30-min incubation period (Fig. 3). After 30 min, less than 3% of the total *cis*-parinaric acid was esterified, with the vast majority of this amount found in the choline- and ethanolamine glycerophospholipids. *Cis*-pari-

TABLE 2
Percent Distribution of [^{3}H]-Oleic Acid in Neutral and Phospholipid Fractions[a]

Lipid species	% Total distribution (dpm/plate)		
	Control cells	High I-FABP	High L-FABP
Nonesterified fatty acids	9.5 ± 0.7	11.5 ± 1.7	10.0 ± 1.0
Cholesteryl esters	9.6 ± 1.1	13.3 ± 1.3[b]	2.7 ± 0.8[c]
Triacylglycerols	2.9 ± 0.3	16.3 ± 3.3[c]	10.9 ± 1.3[c]
Ethanolamine glycerophospholipids	7.1 ± 0.7	10.7 ± 0.3[b]	9.5 ± 0.2[b]
Choline glycerophospholipids	54.6 ± 1.5	39.6 ± 3.1[c]	59.1 ± 0.8[b]
Others	16.3	8.6	7.8

[a]The cells were incubated with [^{3}H]-oleic acid for 30 min and prepared as described in the Materials and Methods section. Listed are the nonesterified fatty acids and the major esterified neutral lipids and phospholipids. "Others" represents the sum total of mono- and diacylglycerols, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, lysophosphatidylcholine, and phosphatidylinositol-4-phosphate. Data represent means ± SEM with n = 3–5. See Table 1 for abbreviations.
[b]$P < 0.05$ from control cells.
[c]$P < 0.001$ from control cells.

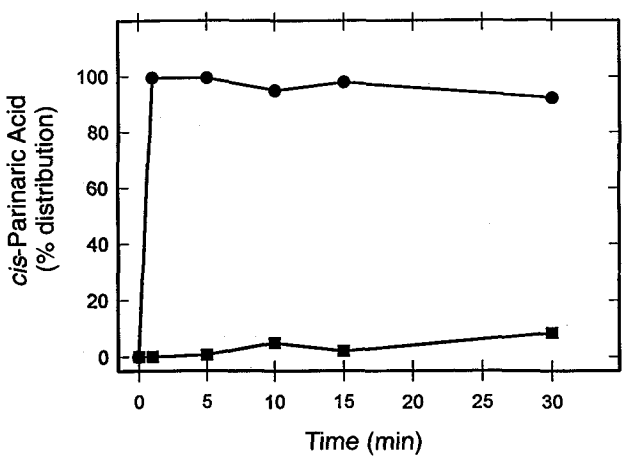


FIG. 3. The percent distribution of *cis*-parinaric acid in L-FABP expressing L-cells. The circles represent total cellular free *cis*-parinaric acid as determined by high-performance liquid chromatography (HPLC). The squares represent *cis*-parinaric acid esterified into phospholipids as determined by HPLC. Data points are the average values from two separate cell culture plates at each time point. Abbreviation as in Figure 1.

naric acid was marginally esterified into triglycerides, with this amount representing less than 0.1% of the total *cis*-parinaric acid taken up. Hence, over 97% of the *cis*-parinaric acid taken up by the cell remains in the free fatty acid pool.

To better illustrate the differential effects of I-FABP and L-FABP on fatty acid trafficking and esterification, the esterified [^{3}H]-oleic acid phospholipid/neutral lipid ratio was calculated for control, I-FABP, and L-FABP expressing cells. This ratio was 3.7 ± 0.4 for control cells, 1.7 ± 0.3 for I-FABP expressing cells, and 5.4 ± 0.5 for L-FABP expressing cells. I-FABP expressing cells had a 220 and 320% decrease in this ratio compared to control cells and L-FABP expressing cells, respectively. In contrast, L-FABP expressing cells had a 44% increase in the phospholipid/neutral lipid ester ratio over control cells.

DISCUSSION

The data presented herein show for the first time that different FABP do not exert the same effect on fatty acid uptake. L-cell fatty acid uptake was resolved from intracellular esterification through the use of the poorly esterified *cis*-parinaric acid. *Cis*-parinaric acid is a naturally-occurring octadecatetraenoic (*cis-trans-trans-cis*) fatty acid that resembles oleic acid in structure and is nonfluorescent in aqueous medium but fluoresces strongly when bound to FABP (3). Because *cis*-parinaric acid is only slightly esterified in L-cells (<3% in 30 min), a fluorescent increase closely represents fatty acid uptake in the absence of appreciable esterification. L-FABP expressing cells had a 53% increase in the initial *cis*-parinaric acid uptake rate and 29% increase in the extent of *cis*-parinaric acid uptake. In contrast, I-FABP had no effect. This indicates that L-FABP expression, but not I-FABP expression, increased the fatty acid flux into the cell.

The bulk of this fluorescent increase stimulated by L-FABP can be accounted for by *cis*-parinaric acid insertion into cellular membranes; although *cis*-parinaric acid bound to the L-FABP could account for some of this increased fluorescence. In assays *in vitro*, L-FABP has a 3.4-fold higher fluorescence yield than I-FABP in the presence of saturating *cis*-parinaric acid levels (3). However, this difference *in vitro* cannot be extrapolated to an intact cell system because the fatty acid can partition into an abundance of membrane relative to the FABP levels.

L-FABP and I-FABP expression differentially affected intracellular [^{3}H]-oleic acid esterification. By determining fatty acid incorporation into specific lipid classes, differences in exogenous fatty acids trafficking by the two proteins were resolved. Cells expressing L-FABP preferentially incorporated [^{3}H]-oleic acid into phospholipids, primarily choline- and ethanolamine glycerophospholipids, rather than esterified neutral lipids (Table 2). In contrast, I-FABP expressing L-cells showed a significant increase in [^{3}H]-oleic acid incorporation into the neutral lipid fraction with a significant decrease

in esterification into the phospholipid fraction. I-FABP expression stimulated a larger increase of [^{3}H]-oleic acid into triacylglycerols and total esterified neutral lipids compared to L-FABP expressing or control cells. Therefore, L-FABP appears to direct newly acquired fatty acids into phospholipids, whereas I-FABP preferentially directs new fatty acids into esterified neutral lipids. The effect of L-FABP may represent increased phospholipid synthesis as seen *in vitro* (8).

Our results support the hypothesis that the two FABP present in the intestine have different roles in fatty acid uptake and metabolism. L-FABP increases fatty acid uptake and targets this fatty acid for esterification into phospholipids, while I-FABP does not increase fatty acid uptake, but does preferentially stimulate esterification into triacylglycerides. The mechanisms by which these differential effects occur are unknown, but may represent the ability of these proteins to stimulate specific synthetic pathways.

ACKNOWLEDGMENTS

The helpful suggestions of Sean Myers-Payne during the preparation of this manuscript were very much appreciated. This work was supported in part by the USPHS National Institutes of Health grant DK41402.

REFERENCES

1. Ockner, R.K., Manning, J.A., and Kane, J.P. (1982) Fatty Acid-Binding Proteins: Isolation from Rat Liver, Characterization, and Immunochemical Quantification, *J. Biol. Chem. 257*, 7872–7878.
2. Alpers, D.H., Strauss, A.W., Ockner, R.K., Bass, N.M., and Gordon, J.I. (1984) Cloning of a cDNA Encoding Rat Intestinal Fatty Acid Binding Protein, *Proc. Natl. Acad. Sci. U.S.A. 81*, 313–317.
3. Nemecz, G., Hubbell, T., Jefferson, J.R., Lowe, J.B., and Schroeder, F. (1991) Interaction of Fatty Acid with Recombinant Rat Intestinal and Liver Fatty Acid Binding Proteins, *Arch. Biochem. Biophys. 286*, 300–309.
4. Lowe, J.B., Sacchettini, J.C., Laposata, M., McQuillan, J.J., and Gordon, J.I. (1987) Expression of Rat Intestinal Fatty Acid-Binding Protein in *Escherichia coli*, *J. Biol. Chem. 262*, 5931–5937.
5. Nemecz, G., and Schroeder, F. (1991) Selective Binding of Cholesterol by Recombinant Fatty Acid-Binding Protein, *J. Biol. Chem. 266*, 17180–17186.
6. Paulussen, R.J.A., and Veerkamp, J.H. (1990) Intracellular Fatty Acid-Binding Proteins: Characteristics and Function, in *Subcellular Biochemistry* (Hilderson, H.J., ed.) Vol. 16, pp. 175–226, Plenum Press, New York.
7. Bass, N.M. (1985) Function and Regulation of Hepatic and Intestinal Fatty Acid-Binding Proteins, *Chem. Phys. Lipids 38*, 95–114.
8. Hubbell, T., Behnke, W.D., Woodford, J.K., and Schroeder, F. (1994) Recombinant Liver Fatty Acid-Binding Protein (L-FABP) Interacts with Fatty Acyl-Coenzyme A, *Biochem. 33*, 3327–3334.
9. Vincent, S.H., and Muller-Eberhard, U. (1985) A Protein of the Z Class of Liver Cytosolic Proteins in the Rat That Preferentially Binds Heme, *J. Biol. Chem. 260*, 14521–14528.
10. Raza, H., Pongubala, J.R., and Sorof, S. (1989) Specific High Affinity Binding of Lipoxygenase Metabolites of Arachidonic Acid by Liver Fatty Acid Binding Protein, *Biochem. Biophys. Res. Comm. 161*, 448–455.
11. Dutta-Roy, A.K., Gopalswamy, N., and Trulzsch, D.V. (1987) Prostaglandin E_1 Binds to Z Protein of Rat Liver, *Eur. J. Biochem. 162*, 615-619.
12. Jefferson, J.R., Powell, D.M., Rymaszewski, Z., Kukowska-Latallo, J., Lowe, J.B., and Schroeder, F. (1990) Altered Membrane Structure in Transfected Mouse L-cell Fibroblasts Expressing Rat Liver Fatty Acid-Binding Protein, *J. Biol. Chem. 265*, 11062–11068.
13. Graham, F.L., and Van der Eb, A.J. (1973) A New Technique for the Assay of Infectivity of Human Adenovirus 5 DNA, *Virology 52*, 456–467.
14. Wigler, M., Sweet, R., Kee Sim, G., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979) Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes, *Cell 16*, 777–785.
15. Hara, A., and Radin, N.S. (1978) Lipid Extraction of Tissues with Low Toxicity Solvent, *Anal. Biochem. 90*, 420–426.
16. Dugan, L.L., Demediuk, P., Pendley II, C.E., and Horrocks, L.A. (1986) Separation of Phospholipids by High Pressure Liquid Chromatography: All Major Classes, Including Ethanolamine and Choline Plasmalogens, and Most Minor Classes, Including Lysophosphatidylethanolamine, *J. Chromatogr. 378*, 317–327.
17. Murphy, E.J., Stephens, R., Jurkowitz, M.S., and Horrocks, L.A. (1993) Acidic Hydrolysis of Plasmalogens Followed by High-Performance Liquid Chromatography, *Lipids 28*, 565–568.

[Received March 27, 1995, and in final revised form July 17, 1995; Revision accepted August 24, 1995]

Effects of Bile Salts on Rat Hepatic Acyl CoA:Cholesterol Acyltransferase

Sandra K. Erickson[a,*] and Paul E.A. Van Zuiden[b]

[a]Department of Medicine, University of California, San Francisco and Department of Veterans Affairs Medical Center, San Francisco, California and [b]Department of Medicine, Brown University and Veterans Administration Medical Center, Providence, Rhode Island

ABSTRACT: Acyl CoA:cholesterol acyltransferase (EC2.3.1.26, ACAT), responsible for intracellular esterification of cholesterol, may play an important role in cholesterol trafficking within the cell, and thus, in maintenance of cellular cholesterol homeostasis. Bile acids are potential regulators of cholesterol trafficking in the liver. Therefore, the effect of bile salts on hepatic ACAT activity was studied in the perfused rat liver. ACAT activity was increased after liver perfusion with either taurocholate or taurochenodeoxycholate. However, addition of these bile salts at physiological concentrations *in vitro* had little effect on microsomal ACAT activity. The increase in hepatic ACAT activity due to perfusion with bile salts was accompanied by reduced accumulation of very low density lipoprotein cholesterol in the perfusate, but there was no effect on 3-hydroxy-3-methylglutaryl–CoA reductase activity. Hepatic ACAT activity was decreased after bile diversion for four hours in the intact animal. This treatment had no statistically significant effect on 3-hydroxy-3-methylglutaryl–CoA reductase activity. These data suggest that bile salts induce changes in hepatic compartmentation and traffic of cholesterol within the hepatocyte accompanied by response of ACAT activity to maintain cellular cholesterol homeostasis.

Lipids 30, 911–915 (1995).

The liver is a key organ for the maintenance of whole body cholesterol homeostasis. It plays an important role in regulating both plasma lipoprotein and biliary lipid metabolism. Maintenance of cholesterol homeostasis within the liver involves a complex interplay among cellular and extracellular factors. The major routes of egress of cholesterol and its metabolites from the liver are in lipoproteins and the bile. These two pathways appear to be regulated interdependently; i.e., perturbations in one can lead to changes in the other (1–6). Based on previous work (7–10), the enzyme acyl CoA: cholesterol acyltransferase (EC2.3.1.26, ACAT) responsible for intracellular sterol esterification could play a pivotal role in the direction of cholesterol movement within the hepatocyte, specifically, into the biliary or lipoprotein secretory pathways or into a cholesterol ester (CE) storage compartment. Thus, factors which affect biliary lipid metabolism, such as bile salts, might be expected to affect hepatic ACAT activity, lipoprotein cholesterol secretion, and cholesterol storage.

Feeding bile salts increases hepatic ACAT activity (11,12); however, such a treatment can alter the amounts of cholesterol reaching the liver; thus, the direct effects of bile salts on hepatic ACAT activity are unknown. The perfused rat liver model was utilized to study the effects of short-term administration of the bile salts, taurocholate and taurochenodeoxycholate, on ACAT activity. These effects were compared with the known response of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase, a rate-limiting enzyme of cholesterol synthesis, to this treatment. Further, the effect of bile salts on the net accumulation of very low density lipoprotein (VLDL) cholesterol and triglyceride accumulation in the perfusate was determined. The influence of loss of bile salts on hepatic ACAT and HMG–CoA reductase activities was evaluated in the intact animal after 4-h bile diversion.

EXPERIMENTAL PROCEDURES

Animals. Male, Sprague-Dawley rats, 180–200 g (Simonsen, Gilroy, CA; or Bantin and Kingman, Newark, CA) were housed under normal illumination (lights on 6 a.m.; lights off 6 p.m.). They were allowed free access to food and water at all times. All protocols were approved by animal welfare committees at Stanford University or at the VA Medical Centers (San Francisco, CA, and Providence, RI).

Liver perfusion. Livers were perfused *in situ* as described previously (13) by the method of Mortimore (14) using a silastic lung as described by Hamilton *et al.* (15). Prior to perfusion, a small liver lobe was removed for assay of preperfusion enzyme values. After establishment of perfusion, 16 μmoles Na taurocholate/100 g rat/h or 23 μmoles Na taurochenodeoxycholate/100 g rat/h were added to the perfusate as described previously by Cooper (16). The bile salts were from Sigma (St. Louis, MO). The perfusate also contained 3% bovine serum albumin (Cohn fraction V; Sigma). No gross deleterious effects on the liver were observed as assessed by liver color, ability to extract 0_2, and absence of perfusate loss.

*To whom correspondence should be addressed at VA Medical Center (111F), 4150 Clement St., San Francisco, CA 94121

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; CE, cholesterol esters; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA, VLDL, very low density lipoproteins.

Copyright © 1995 by AOCS Press

Control rats received an equal volume of vehicle. The perfusate was allowed to recirculate for two hours after which time the perfusate was collected and the livers washed with iced saline. Liver microsomes were prepared (17) and assayed for ACAT (11) and HMG–CoA reductase (17). Perfusate VLDL was isolated by centrifugation at d = 1.019 and assayed for cholesterol and triglyceride as described previously (13).

Bile diversion. Bile was diverted in intact animals by cannulation of the common bile duct and drainage of the bile into a tube. The animals were placed in restraining cages and allowed free access to water. Control animals were sham operated and also placed in restraining cages and allowed free access to water. Bile was collected over 4-h and analyzed as described previously (18). The animals were anesthetized and their livers removed after the 4 h bile diversion and microsomes prepared and assayed for reductase and ACAT as above.

In vitro *effects of bile salts.* Liver microsomes were prepared (17) from untreated rats. Na cholate, Na chenodeoxycholate, Na taurocholate, or Na taurochenodeoxycholate was added to the assay tubes over the concentration range 10^{-9}–10^{-3}M and ACAT activity assayed as above.

Statistical Methods. Student's *t*-test was used to assess significance of group differences after correlation for multiple comparisons.

RESULTS AND DISCUSSION

Hepatic ACAT activity was increased within 2 h after addition to the perfusate of either taurocholate or taurochenodeoxycholate compared with the control perfusions or with the preperfusion values (Table 1). The increase in ACAT activity after bile salt perfusion was unlikely to reflect a change in increased cholesterol availability due to increased *de novo* cholesterol synthesis in as much as Liersch *et al.* (19) reported that *de novo* synthesis of cholesterol was unaffected by perfusion of bile salts. In the present work, perfusion with bile salts had no statistically significant effect on the activity of HMG–CoA reductase, a rate-limiting enzyme for cholesterol synthesis (Table 1), confirming previous observations by Cooper (16), and further supporting the hypothesis that bile salt perfusion had not altered *de novo* synthesized cholesterol available to ACAT.

The increases in ACAT activity were not accompanied by increased recovery of esterified cholesterol in VLDL (Fig. 1). Net accumulation of perfusate VLDL cholesterol and triglyceride was decreased after perfusion with either bile salt. This observation is consistent with that reported by Del Pozo and Barth (20) who observed decreased VLDL secretion by isolated rat hepatocytes exposed to bile salts. The ratio of free to esterified cholesterol in VLDL was unchanged by bile salt perfusion; free cholesterol was about 70% of the total in all samples. Thus, increased CE formation by ACAT induced by bile salt perfusion did not lead to net increase in VLDL CE accumulation in the perfusate.

Although the total amounts of cholesterol recovered in the liver after perfusion in the presence or absence of bile salts were similar [2.34 ± 0.23 mg/g liver (control) vs. 2.38 ± 0.17 mg/g liver (cholate) vs. 2.55 ± 0.08 mg/g liver (chenodeoxycholate), not statistically significantly different, n = 3–4 animals in each group], the proportion as CE increased from about 2.5% in the livers after the control perfusions to approximately 16% (range 8–28%) in the livers after perfusion with either bile salt. Similar observations were reported by Cooper (16). Thus, the increase in ACAT activity coupled with decreased secretion of VLDL cholesterol resulted in increased CE storage in the liver after bile salt perfusion.

Little is known about the effects of bile salt perfusion on VLDL metabolism. In addition to possible effects on secretion, the observed decrease in perfusate VLDL cholesterol and triglyceride accumulation might be due to effects on uptake of VLDL or its remnants by the liver. However, this seems unlikely, since Spady *et al.* (21) found that infusion of bile salts into normal rats for eight hours had no effect on hepatic low density lipoprotein receptor-dependent transport or on cholesterol synthesis. Thus, in the rat, short-term liver perfusion with bile salts would seem unlikely to have induced a change in low density lipoprotein receptor-dependent uptake of newly secreted VLDL.

TABLE 1
Effect of Perfusion with Bile Salts on Hepatic ACAT and HMG–CoA Reductase Activities[a]

Perfusion	ACAT (nmole CE/min/mg prot)	% Increase from preperfusion value	HMG–CoA reductase (nmol MVA/min/mg prot)	% Increase from preperfusion value
Control (11)	0.11 ± 0.01[b]	1 ± 7	0.28 ± 0.04[b]	75 ± 20
+ Cholate (11)	0.17 ± 0.02[c]	57 ± 8[d]	0.46 ± 0.10	119 ± 23
+ Chenodeoxycholate (10)	0.16 ± 0.02[c]	50 ± 9[d]	0.32 ± 0.05	88 ± 20

[a]Rat livers were perfused *in situ* as described in the Experimental Procedures section after removal of a small liver lobe for assay. Liver microsomes were then prepared and assayed as described. The numbers in parentheses are the numbers of animals. All values are ± SEM.

[b]Average preperfusion value for acyl CoA:cholesterol acyltransferase (ACAT) was nmole cholesterol ester (CE)/min/mg protein (prot); the average preperfusion value for 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase was 0.18 ± 0.04 nmoles mevalonate (MVA)/min/mg protein.

[c]Statistically significantly different from control activity after perfusion at $P < 0.01$.

[d]Statistically significantly different from control % increase after perfusion at $P < 0.001$.

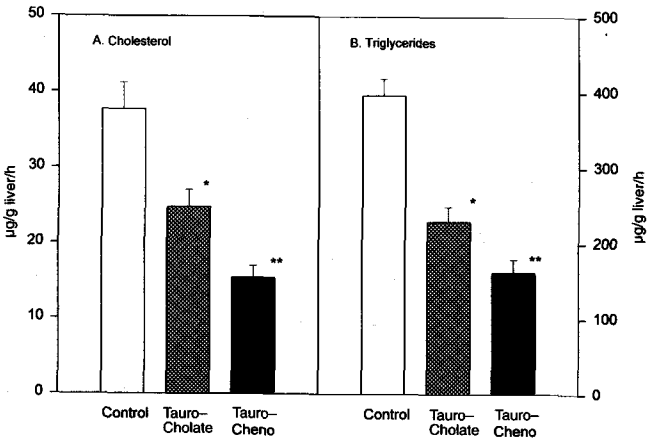


FIG. 1. Effect of taurocholate and taurochenodeoxycholate on net accumulation of very low density lipoprotein cholesterol and triglyceride in rat liver perfusate. Rat livers were isolated and perfused with and without bile salts as described in the Experimental Procedures section. After 2 h, the perfusate was collected and very low density lipoprotein isolated and analyzed as described. The values are the means ± SD. The number of animals is in parentheses. *Different from control at $P < 0.01$; **Different from control at $P < 0.001$. Control (11): open box; + taurocholate (11): hatched box; + taurochenodeoxycholate (10): closed box.

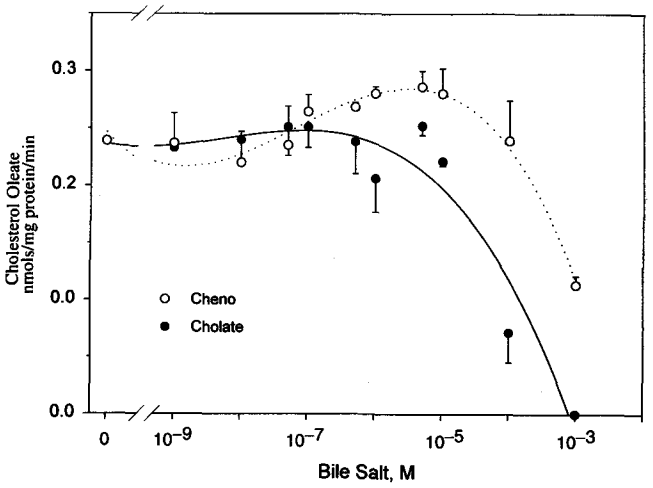


FIG. 2. *In vitro* effects of cholate and chenodeoxycholate on rat liver microsomal acyl CoA:cholesterol acyltransferase activity. The points represent the means ± SD of values obtained using three different preparations of microsomes. Each preparation was assayed in triplicate. Only the values of 10^{-3}M for cholate and 10^{-4} and 10^{-3}M for chenodeoxycholate were statistically significantly different from the values obtained in the absence of bile salt. Cholate, -○-; chenodeoxycholate, -●-.

Administration of bile salts results in increased excretion of cholesterol and phospholipids into the bile (22). Therefore, another explanation for the decreased recovery of VLDL cholesterol and triglyceride induced by bile salt perfusion is that free cholesterol and phospholipid were diverted from the assembly of lipoprotein particles to a pool(s) destined for biliary secretion. Support for this view is provided by the findings of Nervi *et al.* (23) who reported a reciprocal relationship between VLDL cholesterol secretion and the coupling ratio of cholesterol to bile salts in bile.

Because bile salt perfusion increased ACAT activity, it was hypothesized that acute depletion of bile salts might have the opposite effect. However, the short-term effects of bile diversion have not been reported. Longer term studies in intact rats fed cholestyramine, a bile acid-binding resin, revealed that ACAT activity was unchanged (11) or decreased (24), whereas HMG–CoA reductase activity was increased. After acute (4 h) bile diversion in the intact animal, hepatic ACAT activity was decreased about 40% (Table 2). Little change was observed in HMG–CoA reductase activity (Table 2), suggesting that cholesterol was preferentially removed from an ACAT substrate pool which had no regulatory effect on reductase activity.

In order to address the issue of whether bile salts had a direct impact on ACAT activity, their effects were examined in liver microsomes *in vitro*. The physiological range of bile salt concentrations in the normal liver has been estimated to be in the range 0.05–0.3 mM (25,26), reaching a level of 1 mM in cholestasis. No significant changes in ACAT activity were observed over the range 10^{-9}–10^{-5}M for either Na cholate or Na chenodeoxycholate (Fig. 2); however, at concentrations above 10^{-5}M, the activity was inhibited. Similar results were

TABLE 2
Effect of Acute (4 h) Bile Diversion on Rat Hepatic ACAT and HMG–CoA Reductase Activities[a]

Animal	ACAT (nmoles CE/min/mg prot)	HMG–CoA reductase (nmoles MVA/min/mg prot)
Control (3)	0.12 ± 0.01	0.63 ± 0.07
Bile-diverted (3)	0.06 ± 0.02[b]	0.68 ± 0.07

[a]Animals were bile-diverted by cannulation of the common bile duct and placed in restraining cages. Bile was collected. Bile flow was 0.071 ± 0.011 mg/h/g liver, and bile acids were 2.05 ± 0.66 µmoles/h/g liver; cholesterol, 0.029 ± 0.003 µmoles/h/g liver; and phospholipids, 0.15 ± 0.02 µmoles/h/g liver. Control animals were sham operated and also placed in restraining cages. Liver microsomes were prepared 4 h after bile diversion or sham operation and assayed as described in the Experimental Procedures section. The values represent the means ± SE. The number of animals is in parentheses. See Table 1 for abbreviations.

[b]Statistically significantly different from control at $P < 0.05$.

observed with the Na-tauro conjugates, except that significant inhibition was observed only at concentrations above 10^{-3}M (data not shown). Thus, it seems unlikely that bile salts in the physiological concentration range were acting directly on the enzyme or its endogenous membrane cholesterol substrate pool to alter activity *in vivo*, since ACAT activity was increased rather than inhibited after perfusion with bile salts. Reports of bile salt compartmentation in rat liver cells further suggest that bile salts are not acting directly on ACAT in the intact cell. *In vivo*, bile salts in rat liver cells appear to be concentrated in the Golgi apparatus (27), the nuclei (28), and the plasma membrane (29), whereas ACAT activity is localized to the endoplasmic reticulum, with enrichment in the rough fraction (11,30).

Based on the data presented above, we hypothesize that short-term alteration in bile salt traffic also alters the hepatic compartmentation and movement of cholesterol, and that ACAT activity responds rapidly to these changes to maintain cellular cholesterol homeostasis.

ACKNOWLEDGMENTS

We thank Steven Lear for technical assistance and Maggie Cho for typing the manuscript. This work was supported by NIH grant DK38553 and the Department of Veterans Affairs. Some of the experiments were performed in the Division of Gastroenterology, Stanford University School of Medicine (Stanford, CA), and were supported by NIH grant HL05360.

REFERENCES

1. Angelin, B., Einarsson, K., Hellstrom, K., and Leijd, B. (1978) Bile Acid Kinetics in Relation to Endogenous Triglyceride Metabolism in Various Types of Hyperlipoproteinemia, *J. Lipid. Res. 19*, 1004–1016.
2. Angelin, B., Einarsson, K., Hellstrom, K., and Leijd, B. (1978) Effects of Cholestyramine and Chenodeoxycholic Acid in the Metabolism of Endogenous Triglyceride in Hyperlipoproteinemia, *J. Lipid. Res. 19*, 1017–1024.
3. Albers, J.J., Grundy, S.M., Cleary, P.A., Small, D.M., Lachin, J.M., Schoenfield, L.J., and the National Cooperative Gallstone Study Group (1982), The Effect of Chenodeoxycholic Acid on Lipoproteins and Apolipoproteins, *Gastroenterology 82*, 638–646.
4. Grundy, S.M., Ahrens, E.H., Jr., Salen, G., Schreibman, P.H., and Nestel, P.J. (1972) Mechanisms of Action of Clofibrate on Cholesterol Metabolism in Patients with Hyperlipidemia, *J. Lipid Res. 13*, 531–551.
5. Packard, C.J., and Shepherd, J. (1982) The Hepatobiliary Axis and Lipoprotein Metabolism: Effects of Bile Acid Sequestrants and Ileal Bypass Surgery, *J. Lipid Res. 23*, 1081–1098.
6. Portman, O.W., Alexander, M., Tanaka, N., and Osuga, T. (1980) Relationship Between Cholesterol Gallstones, Biliary Function and Plasma Lipoproteins in Squirrel Monkeys, *J. Lab. Clin. Med. 96*, 90–101.
7. Stone, B.G., Erickson, S.K., Craig, W.Y., and Cooper, A.D. (1985) Regulation of Rat Biliary Cholesterol Secretion by Agents That Alter Intrahepatic Cholesterol Metabolism, *J. Clin. Invest. 76*, 1773–1781.
8. Van Zuiden, P.E.A., Cooper, A.D., and Erickson, S.K. (1987), Effects of Lipoprotein Composition on Acyl Coenzyme A:Cholesterol Acyltransferase *in vivo* and in the Perfused Liver and on Hepatic Cholesterol Secretion, *J. Lipid Res. 28*, 930–940.
9. Drevon, C.A., Engelhorn, S.C., and Steinberg, D. (1980) Secretion of Very Low Density Lipoproteins Enriched in Cholesteryl Esters by Cultured Rat Hepatocytes During Stimulation of Intracellular Cholesterol Esterification, *J. Lipid Res. 21*, 1065–1071.
10. Nervi, F.O., Del Pozo, R., Covarrubias, C.F., and Ronco, B.O. (1983) The Effect of Progesterone on the Regulatory Mechanisms of Biliary Cholesterol Secretion in the Rat, *Hepatology 3*, 360–367.
11. Erickson, S.K., Shrewsbury, M.A., Brooks, C., and Meyer, D.J. (1980) Rat Liver Acyl-Coenzyme A:Cholesterol Acyltransferase: Its Regulation *in vivo* and Some of Its Properties *in vitro*, *J. Lipid Res. 21*, 930–941.
12. Heuman, D.M., Vlahcevic, Z.R., Bailey, M.L., and Hylemon, P.B. (1988) Regulation of Bile Acid Synthesis. II. Effect of Bile Acid Feeding on Enzymes Regulating Hepatic Cholesterol and Bile Acid Synthesis in the Rat, *Hepatology 8*, 892–897.
13. Van Zuiden, P.E.A., Erickson, S.K., and Cooper, A.D. (1983) Effect of Removal of Lipoproteins of Different Composition on Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity and Hepatic Very Low Density Lipoprotein Secretion, *J. Lipid Res. 24*, 418–428.
14. Mortimore, G.E. (1961) Effect of Insulin on Potassium Transfer in Isolated Rat Liver, *Am. J. Physiol. 200*, 1315–1319.
15. Hamilton, R.L., Berry, M.N., Williams, M.C., and Sevringhaus, E.M. (1974). A Simple and Inexpensive Membrane "Lung" for Small Organ Perfusion, *J. Lipid Res. 15*, 182–186.
16. Cooper, A.D. (1976) The Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in the Isolated Perfused Rat Liver, *J. Clin. Invest. 57*, 1461–1470.
17. Erickson, S.K., Cooper, A.D., Matsui, S.M., and Gould, R.G. (1977) 7-Ketocholesterol. Its Effects on Hepatic Cholesterogenesis and Its Hepatic Metabolism *in vivo* and *in vitro*, *J. Biol. Chem. 252*, 5186–5193.
18. Erickson, S.K., Lear, S.R., Barker, M.E., and Musliner, T.A. (1990) Regulation of Cholesterol Metabolism in the Ethionine-Induced Premalignant Rat Liver, *J. Lipid Res. 31*, 933–945.
19. Liersch, M.E.A., Barth, C.A., Hackenschmidt, H.J., Ullmann, H.L., and Decker, K.F.A. (1973) Influence of Bile Salts on Cholesterol Synthesis in the Isolated Perfused Rat Liver, *Eur. J. Biochem. 32*, 365–371.
20. Del Pozo, R., and Barth, C.A. (1987) Bile Acids Inhibit Secretion of Very Low Density Lipoprotein by Rat Hepatocytes, *Biol. Chem. Hoppe-Seyler 368*, 887–993.
21. Spady, D.K., Stange, E.F., Bilhartz, L.E., and Dietschy, J.M. (1986) Bile Acids Regulate Hepatic Low Density Lipoprotein Receptor Activity in the Hamster by Altering Cholesterol Flux Across the Liver, *Proc. Natl. Acad. Sci. USA 83*, 1916–1920.
22. Wheeler, H.O., and King, K.K. (1972) Biliary Secretion of Lecithin and Cholesterol in the Dog, *J. Clin. Invest. 50*, 1337–1350.
23. Nervi, F., Marinovic, I., Rigotti, A., and Ulloa, N. (1988) Regulation of Biliary Cholesterol Secretion. Functional Relationship Between the Canalicular and Sinusoidal Cholesterol Secretory Secretory Pathways in the Rat, *J. Clin. Invest. 82*, 1818–1825.
24. Stone, B.G., Evans, C.D., Fadden, R.J., and Schreiber, D. (1989) Regulation of Hepatic Cholesterol Ester Hydrolase and Acyl-Coenzyme A:Cholesterol Acyltransferase in the Rat, *J. Lipid Res. 30*, 1681–1690.
25. Fisher, M.M., Kakis, G., and Yousef, I.M. (1976) Bile Acid Pool in Wistar Rats, *Lipids 11*, 93–96.
26. Yanagisawa, J., Itoh, M., Ishibashi, M., Miyasaki, H., and Nakayano, F. (1980). Microanalysis of Bile Acid in Human Liver Tissue by Selected Ion Monitoring, *Anal. Biochem. 104*, 75–86.

27. Lamri, Y., Roda, A., Dumont, M., Feldmann, G., and Erlinger, S. (1988) Immunoperoxidase Localization of Bile Salts in Rat Liver Cells. Evidence for a Role of the Golgi Apparatus in Bile Salt Transport, *J. Clin. Invest. 82*, 1173–1182.
28. Strange, R.C., Chapman, B.T., Johnston, J.D., Nimmo, I.A., and Percy-Robb, I.W. (1979) Partitioning of Bile Acids into Subcellular Organelles and the *in vivo* Distribution of Bile Acids in Rat Livers, *Biochim. Biophys. Acta. 573*, 535–545.
29. Simion, F.A., Fleischer, B., and Fleischer, S. (1984) Subcellular Distribution of Bile Acids, Bile Salts and Taurocholate Binding Sites in Rat Liver, *Biochem. 23*, 6459–6466.
30. Balasubramanian, S., Venkatesan, S., Mitropoulos, K.A., and Peters, T.J. (1978) The Submicrosomal Localization of Acyl Coenzyme A-Cholesterol Acyltransferase and Its Substrate, and of Cholesterol Esters in Rat Liver, *Biochem. J. 174*, 863–872.

[Received March 7, 1995, and in final revised form July 12, 1995; Revision accepted August 4, *1995*]

The Effects of Simvastatin and Cholestyramine, Alone and in Combination, on Hepatic Cholesterol Metabolism in the Male Rat

John H. Shand and David W. West*

Hannah Research Institute, Ayr, KA6 5HL, United Kingdom

ABSTRACT: The influence of dietary simvastatin, cholestyramine, and the combination of simvastatin plus cholestyramine on hepatic cholesterol metabolism has been investigated in male rats. Recovery from the effects of the drugs was also investigated by refeeding normal chow for 24 h. Both drugs, alone and in combination, increased 3-hydroxy-3-methylglutaryl-CoA (HMG–CoA) reductase activity *in vitro*, but activity returned toward control values, after drug withdrawal. Acyl-CoA:cholesterol acyltransferase (ACAT) was significantly reduced ($P < 0.001$) by simvastatin (–75%), cholestyramine (–71%), and by the drug combination (–81%), due both to a decrease in microsomal cholesterol and to nonsubstrate-dependent modulation of enzyme activity. Refeeding control diet increased ACAT activity but not to control levels. The enhanced activity arose partly from higher microsomal cholesterol and partly from increases in total enzyme activity. Cytosolic neutral cholesteryl ester hydrolase (CEH) activity was substantially elevated by simvastatin (3-fold) and by the drug combination (6-fold), whereas the effect of cholestyramine was smaller (1.5-fold). Normal chow for 24 h only partially returned cytosolic CEH activity to control values. Microsomal CEH activity was increased by simvastatin, alone and in combination with cholestyramine (1.4 to 1.7-fold), and was also enhanced, in the cholestyramine-treated animals, following drug withdrawal. Removal of simvastatin did not allow recovery of this enzyme activity, while withdrawal of the drug combination led to values 29% below controls. The results indicate that in the rat, simvastatin and cholestyramine alter both ACAT and CEH activity, as well as inhibiting HMG–CoA reductase activity.

Lipids 30, 917–926 (1995).

The maintenance of cholesterol balance in the intact liver involves the regulation of several pathways of cholesterol influx and efflux, including the uptake of chylomicron and very low density lipoprotein (VLDL) remnants, the secretion of cholesterol and bile acids into bile, and the synthesis and secretion of lipoproteins as well as storage as cholesteryl esters (CEs) (1). In addition, the liver is critically involved in the maintenance of whole-body sterol balance, since net cholesterol excretion occurs almost exclusively through the hepatobiliary tract.

In most species, inhibition of the rate-determining step in hepatic *de novo* cholesterol synthesis, the reductive cleavage of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) to mevalonate, catalyzed by HMG–CoA reductase, leads to an increase in low density lipoprotein (LDL) receptor numbers and a consequent decrease in plasma LDL cholesterol (2,3). Hence, drug-induced inhibition of HMG–CoA reductase activity is one of the major strategies for dealing with hypercholesterolemia in patients (4–6). Among the drugs that have been clinically approved for this purpose are the statins, lovastatin, simvastatin, and pravastatin, which are specific competitive inhibitors of the enzyme (3–5). However, it is becoming apparent that the mechanism of action of these HMG–CoA reductase inhibitors is more complex than simply the inhibition of cholesterol synthesis, with consequent up-regulation of LDL receptors and increased catabolism of LDL and other apo-B-containing lipoproteins (6). In particular, evidence has been accumulating that one effect of these drugs is to stimulate the induction of HMG–CoA reductase in the cell to restore cholesterol synthesis (7,8).

However, cells do not accumulate large quantities of this compound, and cholesterol, excess to the immediate requirement of the cell, is deposited in the form of cytoplasmic lipid droplets containing insoluble cholesterol–fatty acid esters. These are formed from long-chain acyl-CoA derivatives and cholesterol in a reaction catalyzed by acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) (9). These esters are not inert but can be rehydrolyzed to release the cholesterol for cellular use. Thus, the regulation of hepatic CE metabolism is mediated, in part, by the balance between this esterification by ACAT and hydrolysis of the resultant CEs, by a neutral cholesteryl ester hydrolase (CEH, EC 3.1.1.13) (10,11). Nevertheless, it has been suggested that ACAT plays the major role in this regulation, by providing a defense against excess free cholesterol accumulation (12) with CEH, which includes both cytosolic (soluble) and membrane-associated fractions (11,13) playing only a passive role, and having no importance in maintaining free cholesteryl levels in the hepatocyte (12).

*To whom correspondence should be addressed.

Abbreviations: ACAT, Acyl-CoA:cholesteryl acyltransferase; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; HDL, high density lipoprotein; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; LDL, low density lipoprotein; TAG, triacylglycerols; TRIS, *tris*(hydroxymethyl)-aminomethane; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

Although the mechanisms controlling the activity of these two opposing enzymes are unknown at present, recent evidence has suggested that the statins have major effects on ACAT activity. In the cells of the intestinal wall, ACAT has been implicated in the process of cholesterol absorption from the gut (14–16), and Ishida *et al.* (17,18) found that simvastatin increased fecal excretion of sterol in rabbits fed cholesterol, but not in rabbits fed on normal chow. Kam *et al.* (19) found similar results when the ACAT inhibitor 58-035 (Sandoz, Inc., East Hanover, NJ) was used to inhibit the enzyme activity in human intestinal CaCo-2 cells (supplied by Drs. E. Schaefer and J. Ordovas, Tufts University School of Medicine, NJ). Since cholesterol feeding increases the ACAT levels in intestinal cells, it was postulated that simvastatin was inhibiting the activity of this enzyme.

In a study of VLDL–lipid secretion in perfused livers of rats given lovastatin, Khan *et al.* (20) found that, while hepatic concentrations of free cholesterol were unaltered, the CE content was markedly reduced along with VLDL–lipid production. Lovastatin was also found to inhibit ACAT in, and to decrease the basal secretion of, newly synthesized CE and triacylglycerols (TAG) from Caco-2 cells (21). These authors postulated that this effect of lovastatin on lipid secretion was due to its inhibition of ACAT. They suggested, furthermore, that the results of Kahn *et al.* (20) also could be explained on the basis that lovastatin was regulating CE content and VLDL secretion by its inhibition of ACAT activity and not by its effect on newly synthesized cholesterol, as suggested by Reimann *et al.* (22). Further supporting evidence was provided by Kasim *et al.* (23) who found that lovastatin-treated obese Zucker rats also showed a decline in VLDL secretion and CE content with no change in free cholesterol. Since the substrate of ACAT was freely available, they again raised the possibility that lovastatin may suppress the activity of ACAT.

In contrast, recent investigations have shown that ACAT activity in human liver (24) and in mononuclear leucocyte microsomes (25) was not inhibited by pravastatin, whereas it was increased in diabetic patients following simvastatin treatment (26). Furthermore, Ishida *et al.* (27) demonstrated that simvastatin had a direct inhibitory effect on ACAT in isolated intestinal microsomes although the parent lactone form of the drug rather than the open β-hydroxy form was inhibitory in this system. In contrast, the free hydroxy acid, which is rapidly formed in the liver and in the plasma of rats, is the inhibitor of HMG–CoA reductase in tissues.

Thus, there is controversy concerning the effect of this drug on ACAT, and although simvastatin is reported to have reduced hepatic ACAT in cholesterol-fed rabbits in a preliminary experiment (18), there appears to have been no systematic investigation of the inhibition of hepatic ACAT by the statins.

Cholestyramine is a second type of drug that is used to control plasma cholesterol and LDL levels. This resin sequesters bile acids in the gut, preventing their recirculation to the liver (28,29), one consequence of which is increased diversion of free cholesterol in the liver toward bile acid synthesis. The liver compensates by producing more cholesterol, *via* induction of HMG–CoA reductase (30–32), which then tends to blunt the effectiveness of this agent in reducing plasma cholesterol. Because of this effect, cholestyramine often has been combined with the inhibitors of HMG–CoA reductase in treatment of hypercholesterolemia.

We therefore fed both simvastatin and cholestyramine, alone and in combination, to male rats and investigated the effect of the drugs on a number of parameters of hepatic cholesterol metabolism. Since removal of simvastatin leads to a rapid reversal of the induction of HMG–CoA reductase (33), we also investigated the effect of refeeding these animals with control diet for a short period.

The results indicate that, while these drug treatments influence HMG–CoA reductase activity as expected, they also reduce hepatic ACAT activity and at the same time induce a compensatory increase in the activity of the neutral CEHs in the cell. Refeeding the rats with control diet for 24 h returns HMG–CoA reductase to control levels, but the changes in ACAT and CEH activity require a longer period to overcome the effects of the drugs. These variations in the activities of the cholesterol metabolizing enzymes have major effects on the balance between cholesterol and CE in the cell and plasma.

MATERIALS AND METHODS

Materials. [9,10(n)-^{3}H]Oleic acid (370 GBq/mmol), [1-^{14}C]-oleic acid (2 Gbq/mmol), cholesteryl [1-^{14}C] oleate (2 Gbq/mmol), and DL-3-hydroxy-3-methyl[3-^{14}C]glutaric acid (2 Gbq/mmol) were all obtained from Amersham (Little Chalfont, United Kingdom). RS-[5-^{3}H]Mevalonolactone (750 Gbq/mmol) was purchased from DuPont (Stevenage, United Kingdom). Cholesteryl [9,10(n)-^{3}H]oleate, [9,10(n)-^{3}H]-oleoyl CoA, and 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA were synthesized as described previously (34–36). Dithiothreitol was purchased from Calbiochem (Nottingham, United Kingdom). All other biochemicals were from Sigma (Poole, United Kingdom).

Animals and diets. Male Wistar rats, weighing about 120–140 g at the start of the experiment, were kept on a constant light/dark cycle (lights on 0800–2000 h) and were fed for five days on a crushed CRM(X) breeding diet (Special Diet Services, Manea, Cambridgeshire, United Kingdom) containing 2.4% (w/w) fat and less than 0.01% (w/w) cholesterol. Rats were split into groups and for five days were fed either the control diet, or that same diet supplemented with cholestyramine (2%), simvastatin (0.1%), or a combination of cholestyramine (2%) and simvastatin (0.1%). At the end of the five-day period, the controls and one-half of the drug-treated rats were killed while the remainder of the treated rats were returned to the control diet for 24 h before they were killed. Simvastatin was incorporated by mixing an acetone solution with the crushed diet then allowing the solvent to evaporate for at least 48 h. The control diet was treated in the same way but with acetone alone, and cholestyramine resin was mixed dry into this treated control diet. The rats were fed

at 0830 at the rate of 30 g per rat per day and were weighed at daily intervals. At the end of the experimental period, the animals were killed by cervical dislocation at 0930, i.e., 25 h after their last feed, and the livers were excised, weighed, and frozen in liquid nitrogen. Blood samples were centrifuged immediately (10,000 × *g*) in heparinized tubes and the clear plasmas stored at −20°C.

Tissue processing. Livers were homogenized as described previously (34,37) in 4 vol of ice-cold buffer consisting of 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM *tris*(hydroxymethyl)-aminomethane (TRIS)/HCl buffer, pH 7.2, containing 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-*bis*(2-aminoethyl)tetraacetic acid, 1 mM dithiothreitol, and proteinase inhibitors (1 μg/mL each of pepstatin A, leupeptin, and antipain). Microsomal and cytosolic fractions were then prepared according to the method of Shand *et al.* (34,37) and stored in aliquots at −80° C.

Enzyme assays. The activity of ACAT was assayed in microsomes (150 μg protein) by measuring the rate of conversion of [9,10(n)-^{3}H]oleoyl CoA to cholesteryl [9,10(n)-^{3}H]-oleate as previously described using endogenous cholesterol as the other substrate (35). In addition, parallel assays were performed in the presence of exogenous cholesterol (200 μg/mL) added as a suspension of liposomes, prepared as described by Batzri and Korn (38), using cholesterol and phosphatidylcholine in a molar ratio of 1:2. All assays were preincubated for 30 min before addition of the oleoyl CoA substrate, and incubation time was limited to 5 min to ensure linearity. Cholesteryl [1-^{14}C]oleate was used as an internal standard to correct for recovery.

The activity of neutral CEH was measured, essentially according to Shand and West (34), using 50 μg of either microsomal or cytosolic protein in a total volume of 200 μL. The reaction was initiated by the addition of an ethanolic solution of cholesteryl [9,10(n)-^{3}H]oleate (20 nmol, 1×10^6 dpm) and terminated after 45 min. [1-^{14}C]Oleic acid was then added as a recovery standard, and the rate of hydrolysis of the substrate was measured by estimation of the released [9,10(n)-^{3}H]oleic acid.

The 'total' activity of HMG–CoA reductase was determined using 100 μg of microsomal protein in a final volume of 150 μL according to the method described by Shand and West (36). The reaction was initiated by addition of 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA (22 nmol, 2×10^5 dpm) and terminated after 15 min at 37°C by addition of concentrated HCl (10 μL). A recovery standard of RS-[5-^{3}H]mevalonolactone (30 μmol, 1.5×10^4 dpm) was added and the reaction product separated by thin-layer chromatography.

Assay of the neutral CEH inhibitor protein. The liver cytosols were assayed for inhibitory activity against rat mammary gland microsomal neutral CEH essentially as described previously (34) using 30–40 μg of cytosolic protein. The quantity of cytosolic protein required to produce 50% inhibition of the neutral CEH was then calculated. Controls, without mammary gland microsomes, were incubated at the same time to allow correction for the activity of cytosolic neutral CEH.

Estimation of cholesterol and CEs. Lipids, extracted (39) from the liver homogenates and microsomal suspensions, were redissolved in ethanol while those extracted from plasma lipids were resuspended, by sonication, in buffer containing 50 mM TRIS, pH 7.9, 100 mM KCl, 20 mM KF, and 0.05% (wt/vol) Triton X-100. Total and free cholesterol were then assayed by the fluorometric method of Gamble *et al.* (40) and esterified cholesterol estimated by the difference.

Estimation of TAG. TAG were estimated directly in the liver homogenates and in the aqueous suspensions of the extracted plasma lipids by a modification of the method of McGowan *et al.* (41) using assay kit number 339-10 as supplied by Sigma.

Protein and DNA measurements. Protein concentrations were assayed by the dye-binding method of Bradford (42) and DNA as described by Labarca and Paigen (43).

RESULTS

Diet-induced effects of cholestyramine. Animals fed the cholestyramine diet gained the same weight as controls but exhibited a small (12%) reduction in liver weight. This diet led to a significant increase in liver TAG, whether expressed on a cellular (i.e., related to DNA concentration) or a total liver basis (+23% and +33% respectively, Table 1). CE levels were unaffected whereas cholesterol levels were reduced (−9%, $P < 0.01$) in cellular terms (Table 1). Drug withdrawal led to increases in all of these parameters within 24 h. In contrast, the diet containing cholestyramine had little effect on plasma lipid levels although drug withdrawal led to a 32% drop in plasma cholesterol levels (Table 2).

HMG–CoA reductase activity was increased (1.6-fold) by the cholestyramine treatment (Table 4), as were both microsomal (Table 4) and cytosolic (Table 3) CEH activities, although the increases were much less than was observed with the simvastatin treatment (1.1- and 1.5-fold, respectively, Fig. 1) correlating with a significant decrease (−27%, $P < 0.01$) in the activity of the cytosolic inhibitor of CEH (Table 3). Refeeding normal diet returned HMG–CoA reductase, cytosolic CEH, and inhibitor activity toward normal values but increased the microsomal CEH still further (to 200% of controls). A significant reduction in ACAT activity (−71%, $P < 0.001$, Table 4) was also observed in cholestyramine-treated animals, and this also was observed when the assay was repeated in the presence of exogenously supplied cholesterol. Following drug withdrawal, ACAT activity was significantly raised both when measured with endogenous cholesterol (+54%, $P < 0.001$) and with saturating cholesterol concentrations (+18%, $P < 0.05$), but not sufficiently to return it to the control value. The evidence from the scatter diagram relating ACAT activity to microsomal cholesterol content (Fig. 2) suggests that this enhancement was not due to an increase in the level of microsomal cholesterol.

Diet-induced effects of simvastatin. Animals treated with simvastatin gained considerably less weight during the period of the experiment, and this was reflected in a significant re-

TABLE 1
The Effect of Treatment with Simvastatin and Cholestyramine, Alone and in Combination, on Liver Lipid Levels[a]

	Triacylglycerol		Cholesterol (C)		Cholesteryl esters (CE)		Ratio
Treatment	mg/mg DNA	mg/total liver	μg/mg DNA	mg/total liver	μg/mg DNA	mg/total liver	CE/C in total liver
Control (10)	1.9 ± 0.1	80.9 ± 5.2	315.6 ± 8.5	13.9 ± 0.5	200.2 ± 7.1	8.9 ± 0.5	0.63 ± 0.03
Cholestyramine (6)	2.3 ± 0.1[c]	107.6 ± 4.4[c]	286.6 ± 3.8[c]	13.2 ± 0.5	187.7 ± 3.6	8.7 ± 0.4	0.66 ± 0.02
Cholestyramine + 24-h control diet (6)	2.6 ± 0.1[d]	111.7 ± 3.4	371.3 ± 7.6[d]	16.1 ± 0.9	266.0 ± 5.8[d]	11.5 ± 0.6[c]	0.72 ± 0.02
Simvastatin (6)	1.9 ± 0.1	70.7 ± 5.0	211.7 ± 15.3[d]	7.7 ± 0.5[d]	152.6 ± 7.0[d]	5.4 ± 0.2[d]	0.73 ± 0.03
Simvastatin + 24-h control diet (6)	2.1 ± 0.1	88.4 ± 2.9	246.3 ± 11.3[d]	10.0 ± 0.5[c,e]	199.4 ± 8.2[e]	8.1 ± 0.3	0.81 ± 0.03[c]
Simvastatin + cholestyramine (10)	2.0 ± 0.6	86.5 ± 3.8	318.3 ± 7.7	13.6 ± 0.6	220.9 ± 11.3	9.5 ± 0.7	0.68 ± 0.03
Simvastatin + cholestyramine + 24-h control diet (10)	2.0 ± 0.5	97.8 ± 4.1[b]	338.4 ± 5.0[b]	16.1 ± 0.6[b,e]	354.7 ± 13.5[d,f]	16.7 ± 0.4[d]	1.05 ± 0.03[d,f]

[a]Liver triacylglycerol levels were determined directly in the tissue homogenates, whereas liver cholesterol and cholesteryl esters were measured by means of a fluorometric assay in lipid extracts, dissolved in ethanol, as described in the Materials and Methods section. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values (*b,c,d*) and between treatments and treatments plus 24 h on control diet (*e,f*) were determined using Student's *t*-test. $^{b}P < 0.05$. $^{c,e}P < 0.01$. $^{d,f}P < 0.001$.

duction (7.1 ± 0.1 g to 5.8 ± 0.2 g) in their liver weights. They regained much of their lost body and liver weight after refeeding normal diet. The drug had little effect on the levels of TAG in the plasma (Table 2) and the liver (Table 1), but significantly reduced the cholesterol and the CE content of both plasma and liver, as well as that measured in the microsomal fraction of the liver (Table 5). Drug withdrawal allowed the microsomal and the plasma levels of both lipids to return to normal, whereas hepatic free cholesterol levels remained depressed. HMG–CoA reductase activity was markedly increased by simvastatin treatment (Table 4) as was CEH activity, both microsomal (Table 4) and cytosolic (Table 3). The proportionate increase for the cytosolic CEH was, nevertheless, greater than that for the microsomal CEH (3.0- and 1.7-fold respectively, Fig. 1). In contrast, the activity of the inhibitor of CEH was unaffected (Table 3), whereas ACAT activity, measured both in the presence of endogenous and exogenous cholesterol, was dramatically reduced (–75%, Table 4). Feeding normal chow for 24 h allowed the activity of HMG–CoA reductase to decline toward control values but did not cause any further alteration in the elevated activities of the two CEHs. ACAT activity measured under conditions of saturating cholesterol was unaltered following drug withdrawal, whereas it was increased in the presence of endogenous cholesterol. The scatter diagram (Fig. 2) indicates that this stimulation in ACAT was solely due to increased availability of substrate.

Diet-induced effects of cholestyramine plus simvastatin. The livers, obtained from the animals fed these combined drugs,

TABLE 2
The Effect of Treatment with Cholestyramine, Alone and in Combination with Simvastatin, on Plasma Lipid Levels[a]

Treatment	Triacylglycerol (mg/100 mL)	Cholesterol (C) (mg/100 mL)	Cholesterol ester (CE) (mg/100 mL)	Ratio CE/C
Control (6)	47.2 ± 6.5	17.6 ± 1.4	22.7 ± 1.5	1.31 ± 0.08
Cholestyramine (6)	53.7 ± 10.4	16.8 ± 1.7	19.2 ± 1.4	1.16 ± 0.08
Cholestyramine + 24-h control diet (6)	57.9 ± 3.0	12.0 ± 0.3[c]	22.6 ± 1.1	1.87 ± 0.06
Simvastatin (6)	48.5 ± 3.2	10.2 ± 0.5[d]	16.5 ± 0.8[c]	1.63 ± 0.07
Simvastatin + 24-h control diet (7)	47.8 ± 1.1	15.1 ± 0.7	22.7 ± 1.4	1.51 ± 0.08
Simvastatin + cholestyramine (10)	28.4 ± 2.7[b]	24.8 ± 1.8[c]	20.7 ± 1.5	0.88 ± 0.07
Simvastatin + cholestyramine + 24-h control diet (10)	35.5 ± 5.2	13.3 ± 1.1[b,f]	14.7 ± 1.1[d,e]	1.16 ± 0.05

[a]Plasma triacylglycerols and total cholesterol were estimated in lipid extracts of plasma resuspended in buffer as described in the Materials and Methods section. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values (*b,c,d*) and between treatment and treatment plus 24 h on control diet (*e,f*) were determined using Student's *t*-test. $^{b}P < 0.05$. $^{c,e}P < 0.01$. $^{d,f}P < 0.001$.

TABLE 3
The Effects of Treatment with Simvastatin and Cholestyramine, Alone and in Combination, on the Activities of the Cytosolic Neutral Cholesteryl Ester Hydrolase and the Cytosolic Protein Inhibitor of Neutral Cholesteryl Ester Hydrolase[a]

	Cytosolic CEH		Cytosolic CEH inhibitor activity		"True" cytosolic CEH activity (allowing for inhibitor activity)	
Treatment	pmol/min/ mg protein	nmol/min/ total liver	% inhibition by 50 µg protein	50% inhibitory units/total liver ($\times 10^3$)	pmol/min/ mg protein	nmol/min/ total liver
Control (8)	13.2 ± 0.6	11.6 ± 0.8	47.8 ± 1.5	16.4 ± 1.1	24.5 ± 0.5	21.7 ± 1.4
Cholestyramine (6)	20.2 ± 1.4^{d}	17.6 ± 1.2^{c}	34.9 ± 3.4^{c}	12.1 ± 1.1^{b}	31.2 ± 1.9^{c}	27.1 ± 2.1
Cholestyramine + 24-h control diet (6)	14.9 ± 2.7	13.5 ± 2.3	51.3 ± 4.9	18.7 ± 1.7	31.9 ± 5.7	28.7 ± 4.8
Simvastatin (6)	52.4 ± 2.8^{d}	32.7 ± 2.4^{d}	44.3 ± 1.9	11.0 ± 0.7^{c}	95.2 ± 6.6^{d}	59.2 ± 5.0^{d}
Simvastatin + 24-h control diet (6)	39.7 ± 1.9^{d,e}	28.6 ± 1.9^{d}	46.5 ± 3.7	11.7 ± 2.4	76.4 ± 6.3^{d}	55.8 ± 6.1^{d}
Simvastatin + cholestyramine (8)	77.4 ± 4.3^{d}	61.0 ± 4.2^{d}	37.8 ± 1.6^{c}	12.1 ± 0.8^{c}	124.9 ± 6.8^{d}	98.6 ± 6.2^{d}
Simvastatin + cholestyramine + 24-h control diet (8)	42.0 ± 2.4^{d,f}	44.8 ± 1.4^{d}	45.5 ± 2.2	19.3 ± 0.6^{b}	79.2 ± 7.2^{d}	83.5 ± 4.9^{d}

[a]Cytosolic neutral cholesteryl ester hydrolase (CEH) activity was determined using 50 µg of cytosolic protein incubated with cholesteryl [9,10-^{3}H]oleate for 45 min. The CEH inhibitor protein was assayed by determination of the CEH activity in rat mammary gland microsomes (100 µg protein) in the absence and presence of 30–50 µg of cytosolic protein. The activity in whole liver is expressed as 50% inhibitory units where one unit is the amount (µg) required to inhibit the mammary microsomal CEH by 50%. From an estimate of the percentage inhibition of the CEH activity due to 50 µg, the "true" cytosolic CEH activities could be recalculated to allow for the effect of the inhibitory protein. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values (*b,c,d*) and between treatments and treatments plus 24 h on control diet (*e,f*) were determined using Student's *t*-test. $^{b}P < 0.05$. $^{c,e}P < 0.01$. $^{d,f}P < 0.001$.

weighed 25% less than either controls or animals similarly treated but refed control diet for 24 h after drug withdrawal. Plasma TAG were reduced by the cholestyramine plus simvastatin treatment (–40%, Table 2), whereas hepatic TAG were unaltered (Table 1). Refeeding normal diet for 24 h did not restore plasma TAG to normal levels but did lead to a significant increase (+21%, $P < 0.05$) in the levels of hepatic TAG.

Hepatic free cholesterol levels were unaffected by this drug combination (Table 1) while cholesterol levels in the plasma (Table 2) and in the microsomes (Table 5) reacted in opposition, being increased in the plasma (+41%) but reduced in the microsomes (–16%). CE levels in the plasma and in the whole liver were not significantly affected by this treatment, whereas the microsomal content of esterified cholesterol was

TABLE 4
The Effect of Treatment with Simvastatin and Cholestyramine, Alone and in Combination, on the Activities of the Microsomal Enzymes Acyl-CoA:Cholesterol Acyltransferase, HMG–CoA Reductase, and Neutral Cholesteryl Ester Hydrolase[a]

	Acyl-CoA:cholesterol acyltransferase						
	Endogenous cholesterol			HMG–CoA reductase		Cholesteryl ester hydrolase	
Treatment	pmol/min/ mg protein	pmol/min/ mg protein	nmol/min/ total liver	pmol/min/ mg protein	nmol/min/ total liver	pmol/min/ mg protein	nmol/min/ total liver
Control	368.9 ± 25.4	558.1 ± 8.9	35.2 ± 3.2	399.4 ± 50.4	37.5 ± 4.1	137.6 ± 7.1	12.9 ± 0.5
Cholestyramine	106.3 ± 6.7^{d}	221.9 ± 10.7^{d}	9.5 ± 0.8^{d}	644.1 ± 52.2^{c}	57.4 ± 5.5^{b}	152.1 ± 7.1	13.5 ± 0.7
Cholestyramine + 24-h control diet	163.7 ± 7.3^{d,i}	262.5 ± 11.6^{d,g}	20.6 ± 2.1^{c}	347.1 ± 36.2^{i}	15.8 ± 1.9^{d,i}	182.9 ± 4.0^{d,i}	22.8 ± 1.5^{d,i}
Simvastatin	93.6 ± 9.6^{d}	261.4 ± 19.5^{d}	8.1 ± 0.6^{d}	1487.7 ± 84.8^{d}	130.6 ± 6.8^{d}	236.1 ± 5.6^{d,f}	20.8 ± 0.8^{d}
Simvastatin + 24-h control diet	150.9 ± 23.6^{d,g}	251.3 ± 26.5^{d}	13.5 ± 2.0^{d}	678.6 ± 105.7^{b}	61.2 ± 10.1^{b}	233.7 ± 8.1^{d}	20.9 ± 1.0^{d}
Simvastatin + cholestyramine	70.0 ± 10.1^{d}	150.3 ± 20.0^{d,i}	7.4 ± 1.2^{b}	1683.5 ± 107.9^{d}	174.0 ± 21.1^{d}	186.1 ± 15.8^{b,f}	19.3 ± 2.7^{b}
Simvastatin + cholestyramine + 24-h control diet (4)	247.9 ± 30.0^{b,h}	378.6 ± 31.6^{d}	22.1 ± 1.8^{c}	387.0 ± 56.5^{i}	33.9 ± 3.4^{i}	98.0 ± 4.4^{c,g}	8.9 ± 0.3^{d}

[a]Acyl-CoA:cholesterol acyltransferase was assayed using 150 µg microsomal protein incubated with [9,10-^{3}H]oleoyl CoA for 5 min. Activity was measured using the cholesterol endogenous to the microsomes and in the presence of saturating amounts of exogenous cholesterol added as cholesterol:phosphatidylcholine liposomes as described in the Materials and Methods section. 3-Hydroxy-3-methylglutaryl (HMG)–CoA reductase activity was measured by incubation of 100 µg of protein with 3-hydroxy-3-methyl[3-^{14}C]-CoA for 15 min. Neutral cholesteryl ester hydrolase activity was estimated by incubating 50 µg microsomal protein with cholesteryl [9,10-^{3}H]oleate for 45 min. The results are the means ± SEM for six animals except where indicated by the number in parentheses. Significant differences from control values (*b,c,d*), between treatments (*e,f*) and between treatments and treatments plus 24 h on control diet (*g,h,i*) were determined using Student's *t*-test. $^{b,g}P < 0.05$. $^{e}P < 0.02$. $^{c,h}P < 0.01$. $^{d,f,i}P < 0.001$.

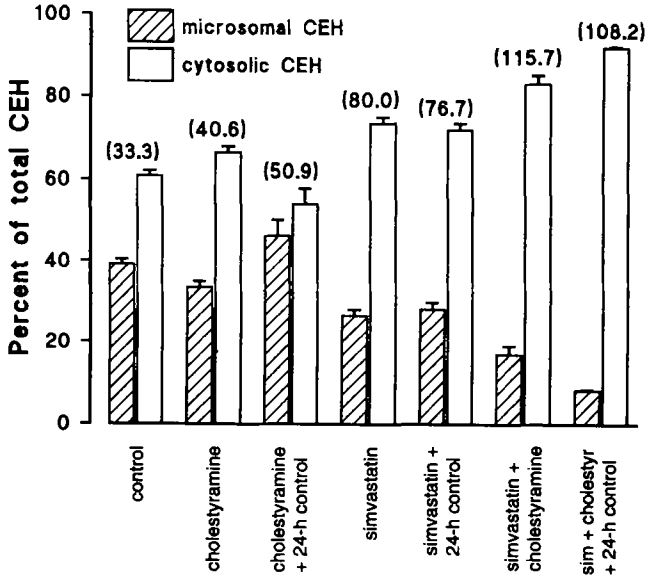


FIG. 1. Histogram showing the relative proportions of the total cholesteryl ester hydrolase (CEH) activity in the cytosolic and in the microsomal fractions of the livers (nmol/min/total liver) following drug treatment. Cytosolic CEH activity was calculated making allowance for the activity of the inhibitory protein of CEH in the appropriate cytosols; sim, simvastatin; cholestyr, cholestyramine.

TABLE 5
The Effects of Treatment with Simvastatin and Cholestyramine, Alone and in Combination, on the Microsomal Contents of Cholesterol and Cholesteryl Esters[a]

Treatment	Cholesterol (C) (μg/mg protein)	Cholesteryl esters (CE) (μg/mg protein)	Ratio CE/C
Control (8)	31.8 ± 0.9	19.6 ± 0.6	0.60 ± 0.02
Cholestyramine (6)	24.1 ± 0.7[d]	16.2 ± 0.7[c]	0.67 ± 0.02
Cholestyramine + 24-h control diet (6)	21.6 ± 0.7[d]	13.6 ± 0.4[d]	0.63 ± 0.01
Simvastatin (6)	27.6 ± 1.1[b]	13.0 ± 0.5[d]	0.47 ± 0.02[d]
Simvastatin + 24-h control diet (6)	33.4 ± 0.7[e]	20.4 ± 0.7[f]	0.61 ± 0.02
Simvastatin + cholestyramine (10)	26.6 ± 0.9[d]	16.1 ± 0.6[d]	0.61 ± 0.01
Simvastatin + cholestyramine + 24-h control diet (10)	26.1 ± 0.5[d]	23.0 ± 0.7[c,f]	0.88 ± 0.03[d]

[a]Cholesterol and cholesteryl esters were determined by means of a fluorometric assay on the extracted microsomal lipids in ethanolic solution. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values (*b,c,d*) and between treatments and treatments plus 24 h on control diet (*e,f*) were determined using Student's *t*-test. [b]$P < 0.05$. [c,e]$P < 0.01$. [d,f]$P < 0.001$.

significantly lowered (−17%, $P < 0.001$). Withdrawal of the drugs led, within 24 h, to an increase in the cholesterol level in the whole liver (+16%) but reversed the increase in plasma free cholesterol (to 24% below control) without affecting microsomal levels. In contrast, hepatic and microsomal CE content was significantly increased (+77 and +18%, respectively) following reversion to the normal diet, and this was reflected in a decrease (−35%) in the CE of the plasma.

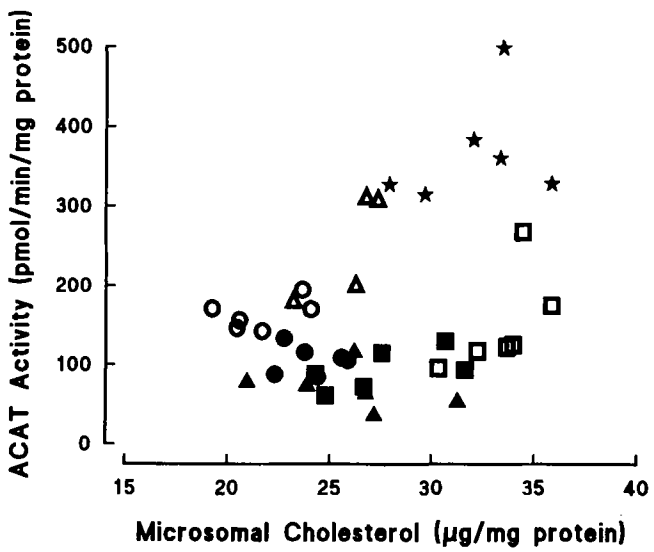


FIG. 2. Scatter diagram relating acyl-CoA:cholesterol acyltransferase (ACAT) activities to microsomal contents of cholesterol. Controls (★), and animals treated with simvastatin (□,■), cholestyramine (○,●) and a combination of the two drugs (△,▲). Open symbols represent drug treatments, filled symbols represent drug-treated animals refed control diet for 24 h.

Feeding both drugs had major effects on the activities of the enzymes of cholesterol metabolism. HMG–CoA reductase was higher (4.2-fold), and ACAT activity, measured both with and without exogenous cholesterol, was significantly reduced (−73 and −81%, respectively, $P < 0.001$), both effects being greater than was observed with simvastatin alone (Table 4). Refeeding control diet for 24 h returned HMG–CoA reductase activities to that of the controls and markedly increased ACAT activities (3.5-fold), which nevertheless remained substantially below control levels. The data in Figure 2 indicates that the enhanced ACAT activity, following drug withdrawal, occurred through a combination of nonsubstrate modulation of enzyme activity, together with an increase in substrate availability.

Cytosolic CEH was dramatically increased (5.9-fold) by this combined drug treatment (Table 3), and this was correlated with a significant reduction (−21%, $P < 0.01$) in the activity of the cytosolic inhibitory protein. Microsomal CEH was also increased, although to a lesser extent (1.4-fold, Table 4), as was emphasized in Figure 1. Withdrawal of the drugs reduced microsomal CEH to below control values, but cytosolic CEH remained substantially elevated (3.8-fold). Allowance for the effect of the cytosolic inhibitor of CEH activity made a quantitative difference but did not alter the relative effects of the drugs (Table 3). It did, however, emphasize that the relative proportions of cytosolic to microsomal activity were markedly higher in the refed animals compared with the drug-treated animals (9.4:1 and 5.1:1, respectively, Fig. 1).

DISCUSSION

The results obtained in the present investigation confirm that the effects of the cholesterol-lowering drugs simvastatin and cholestyramine, alone and in combination, on hepatic cholesterol metabolism are much more complex than simply the inhibition of HMG–CoA reductase with a secondary increase in LDL receptors (4). They clearly cause major variations in the hepatic activities of ACAT and the neutral CEHs which lead to changes in the levels of cholesterol, CEs, and TAG, both in the liver and the plasma of these animals. Moreover, it is clear both from previously published results (44–46) and from the data presented here that the variations in CEH activity, arising from the different dietary conditions, may have an equal importance with ACAT in maintaining intracellular cholesterol levels.

Cholestyramine administration, by promoting fecal loss of bile salts (28,29), creates a cellular need for free cholesterol to replace cholesterol utilized for bile salt synthesis (30) which is apparently met by induction of microsomal HMG–CoA reductase activity (30–32; Table 4) and by inhibition of its degradation (47). However, our data indicate that this free cholesterol requirement also could be partially met by a decrease in CE formation, through a reduction in ACAT activity (Table 4), or an increase in CE hydrolysis, *via* enhancement of CEH activity (Table 4). The significant variation in ACAT was in agreement with previous results (12), whereas the significant increase in cytosolic, but not microsomal, CEH activity contrasted with previous reports (12,48). The change in ACAT appeared to occur partially from a decline in the amount of microsomal cholesterol (Table 5) and partially from a decline in either the amount, or the activity, of the enzyme as indicated by the 50% loss of activity in the presence of saturating concentrations of cholesterol (Table 4). Consideration of the scatter diagram relating ACAT activity to microsomal cholesterol content (Fig. 2) also would suggest that increases in the amount or activity of the enzyme, rather than substrate availability, was the explanation for the enhanced ACAT activity observed when simvastatin was withdrawn. The net result of these changes in enzyme activity was a significant decrease in microsomal cholesterol and CE content although the total hepatic content of these sterols was unaltered. However, at the present stage, it is not possible to apportion these effects between the various enzymes involved.

The major reason for the variation between our results and those previously published (12) in relation to CEH activity arises from the different conditions that were used in the assay. The hepatic cytosol contains a protein inhibitor of this enzyme activity (34) for which allowance can be made by performing the assay with small (50 μg) amounts of protein. Stone *et al.* (12) employed much larger quantities of cytosolic protein, and under the conditions of their assay, it can be assumed that less than 10% of the CEH activity was measurable. Hence, variations in this enzyme activity, due to dietary manipulation, could not be observed.

Simvastatin administration also stimulates the induction of HMG–CoA reductase (47) but, at the same time, leads to a decrease in the activity of ACAT, thus allowing unesterified cholesterol to enter the free cholesterol pool (Table 4). A reduction in hepatic ACAT activity in rats due to simvastatin has not been reported previously, although such a reduction has been proposed to explain a selective decrease in hepatic CE content (23), and lovastatin has been shown to inhibit ACAT activity in intestinal cells (18,21). Microsomal cholesterol was, however, only reduced by 14% below controls, suggesting that the decline (–75%) in ACAT activity was only partially due to decreased substrate availability and was predominantly due to a nonsubstrate modulation of enzyme activity. This interpretation was supported by the 53% decrease in ACAT activity observed in the presence of saturating concentrations of cholesterol. Perhaps more significantly in this situation, both microsomal and cytosolic CEH activities were substantially increased (Tables 3 and 4). The combined effect of reduced ACAT and increased CEH was a significant decline in both total and microsomal CE. Nevertheless, in spite of these compensatory variations in HMG–CoA reductase, ACAT, and CEH, hepatic cholesterol content was still markedly reduced below control values. This may result from increased disposal of cholesterol *via* the biliary route since administration of an HMG–CoA reductase inhibitor, both with (49) and without (50) the addition of a second drug (AOMA, a copolymer of maleic acid and an α-olefin of 18 carbon chains; Monsanto, St. Louis, MO) to block intestinal cholesterol absorption, prompted an increased output of cholesterol to bile. Moreover, since increased biliary cholesterol secretion also has been reported to occur when ACAT activity was inhibited by progesterone (51,52), the reduction in ACAT observed in the present investigation would also be consistent with increased biliary sterol excretion.

The rapid decline in HMG–CoA reductase activity following withdrawal of the simvastatin correlates with the known short half life of this enzyme (47). However, simvastatin is eliminated from the plasma even more quickly (5), and hence, one effect of withdrawal of the drug is a large overshoot in cholesterol production (33). This expanded pool of newly synthesized cholesterol can enrich the hepatic microsomal membrane fraction, part of which can be esterified by ACAT, as indicated by the increased enzyme activity in this fraction (Table 4). Moreover, since the total ACAT activity, measured under saturating substrate conditions, was unaltered, it can be inferred that the observed enhancement of ACAT activity was solely due to the increased substrate availability (Fig. 2) rather than any change in other factors capable of modulating enzyme activity, such as covalent modification (53) or variation in the amount of enzyme protein. Since total ACAT activity was not increased, there appeared to be a limit to the proportion of the excess cholesterol that could be converted to the ester, and this was further constrained by the high CEH activity, which showed negligible response to withdrawal of simvastatin. The inability to further increase the hepatic stores of CEs could help to explain the marked in-

crease in the rate of biliary sterol secretion reported by Bilhartz *et al.* (33).

The marked induction of HMG–CoA reductase activity which results from the diet containing simvastatin and cholestyramine (7,8,30–32) was confirmed by the data presented here. The reduction in ACAT activity following this treatment, which was even greater than that observed in response to the individual drugs, ensured that newly synthesized cholesterol arising from the induced HMG–CoA reductase was not converted to the ester. This strategy was supported by the greater than fivefold increase in cytosolic CEH activity, the net result of which should have been increased free cholesterol levels in the cell. However, in spite of these changes in ACAT and CEH, hepatic cholesterol and CE remained at the same level as in untreated animals, and microsomal CE was only slightly reduced. Major changes had, nevertheless, occurred in the levels of plasma cholesterol, which were substantially elevated, suggesting that increased export of cholesterol to the plasma may have contributed to this maintenance of intracellular sterol levels. A rise in serum cholesterol levels has been observed in rats fed lovastatin for seven days (50), and an increase in high-density lipoprotein (HDL) cholesterol was reported in rats in which hepatic ACAT activity had been inhibited (54). In contrast, Mitchell *et al.* (55) reported a reduction in plasma cholesterol in rats fed this drug combination. The difference between our results and those of Mitchell *et al.* (55) could be explained by the fact that their animals were fasted overnight, a period when they normally would be consuming the bulk of their food, whereas the animals in our study and in that reported in (50) had access to food up to the time of slaughter.

Nevertheless, both Mitchell *et al.* (55) and ourselves observed a fall in plasma TG. They attributed this partly to decreased secretion of VLDL, arising from a decline in the hepatic content of mRNA species coding for apo-B, and partly to upregulation of the B/E receptor, leading to increased clearance. Interestingly, a fall in plasma TAG was also recorded (54) when hepatic ACAT was inhibited, and others have shown that inhibition of ACAT selectively decreased the plasma content of apo-B containing lipoproteins and shifted the distribution of cholesterol to HDL (56). Thus, the fact that ACAT activity was inhibited by both simvastatin and cholestyramine also would support the suggestion of reduced TAG secretion.

As expected, HMG–CoA reductase activity was reduced to control levels following withdrawal of the drugs but, in contrast to the situation with simvastatin alone, total ACAT activity, i.e., measured with saturating substrate, was markedly increased. In these animals, ACAT would have esterified a greater proportion of the expanded pool of free cholesterol, and this, together with the reduced CEH activity present in the microsomal fraction, resulted in a significant increase in microsomal CEs. Nevertheless, the data indicate that, although cytosolic CEH activity remained substantially elevated, there was an accumulation of both cholesterol and CE in the liver. This correlated with significant reductions in the plasma level of these sterols, which could arise from either decreased secretion of lipoproteins and/or increased clearance from the circulation. The increase in the plasma levels of TAG after drug withdrawal would argue against a further reduction in VLDL secretion, particularly as there was an accumulation of hepatic CEs, which have been implicated as a requirement for VLDL secretion (51). Increased clearance is the more likely cause, arising from an upregulation of HDL receptors, since cholesterol loading of cells, that can utilize HDL particles, has been shown to have such effects on plasma sterol levels (57). Furthermore, although this drug combination has been shown to decrease hepatic TAG lipase activity, it resulted in increased levels of the mRNA species coding from this lipase (58). A rapid rise in hepatic TAG lipase levels on drug withdrawal also could contribute to the lowered plasma sterol levels observed.

The cause of the variations in enzyme activity are unknown at present, although available evidence would argue against direct action of the drugs on the enzymes. Cholestyramine remains in the gut and does not enter the circulation, and although the lactone form of simvastatin has been reported to directly inhibit intestinal ACAT, *in vitro*, the open β-hydroxy acid form of the drug was ineffective (27). Since the lactone is rapidly converted into the hydroxy acid in both rat plasma (5) and liver (59–61), it is unlikely that much of the lactone form exists within the circulation.

The results presented here are important since they demonstrate that manipulation of cholesterol synthesis in the rat hepatocyte has major effects on all of the enzymes involved in hepatic cholesterol metabolism, not just on the rate-determining enzyme of cholesterol synthesis. Whether such changes also occur in humans is a subject worthy of investigation.

ACKNOWLEDGMENTS

The authors would like to acknowledge the generous donation of simvastatin by Dr. M. Mark (Karl Thomae GMBH, Biberach, Germany). Financial support was supplied by the Scottish Office Agriculture and Fisheries Department.

REFERENCES

1. Turley, S.D., and Dietschy, J.M. (1982) in *The Liver: Biology and Pathobiology* (Arias, I., Popper, H., Schachter, D., and Shafritz, D.A., eds.) pp. 467–492, Raven Press, New York.
2. Goldstein, J.L., and Brown, M.S. (1990) Regulation of the Mevalonate Pathway, *Nature 343*, 425–430.
3. Davignon, J., Montigny, M., and Dufour, R. (1992) HMG-CoA Reductase Inhibitors: A Look Back and a Look Forward, *Can. J. Cardiol. 8*, 843–864.
4. Feussner, G. (1994) HMG CoA Reductase Inhibitors, *Curr. Opin. Lipidol. 5*, 59–68.
5. Blum, C.B. (1994) Comparison of Properties of Four Inhibitors of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, *Am. J. Cardiol. 73*, 3D–11D.
6. Hunninghake, D.B. (1992) HMG CoA Reductase Inhibtors, *Curr. Opin. Lipidol. 3*. 22–28.
7. Chin, D.J., Luskey, K.L., Anderson, R.G.W., Faust, J.R., Goldstein, J.L., and Brown, M.S. (1982) Appearance of Crystalloid Endoplasmic Reticulum in Compactin-Resistant Chinese Hampster Cells With a 500-Fold Increase in 3-Hydroxy-3-Methylglu-

taryl Coenzyme A Reductase, *Proc. Natl. Acad. Sci. USA. 79*, 1185–1189.
8. Roitelman, J., and Shechter, I. (1986) Altered Kinetic Properties of Rat Liver 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Following Dietary Manipulations, *J. Biol. Chem. 261*, 5061–5066.
9. Chang, T.Y., and Doolittle, G.M. (1983) Acyl Coenzyme A:Cholesterol O-Acyltransferase, in *The Enzymes*, 3rd edn. (Boyer, P., ed.) Vol. 16, pp. 523–539, Academic Press, New York,
10. Dekin, D., and Goodman, D.S. (1962) The Hydrolysis of Long-Chain Fatty Acid Esters with Rat Liver Enzymes, *J. Biol. Chem. 237*, 3649–3656.
11. Nilsson, A. (1976) Hydrolysis of Chyle Cholesterol Esters With Cell-Free Preparations of Rat Liver, *Biochim. Biophys. Acta 450*, 379–389.
12. Stone, B.G., Evans, C.D., Fadden, R.J., and Scheiber, D. (1989) Regulation of Hepatic Cholesterol Ester Hydrolase and Acyl-Coenzyme A:Cholesterol Acyltransferase in the Rat, *J. Lipid Res. 30*, 1681–1690.
13. Erickson, S.K., Lear, S.R., and McCreery, M.J. (1994) Functional Sizes of Hepatic Enzymes of Cholesteryl Ester Metabolism Determined by Radiation Inactivation, *J. Lipid Res. 35*, 763–769.
14. Heider, J.G., Pickens, C.E., and Kelly, L.A. (1983) Role of Acyl-Coenzyme A:Cholesterol Acyltransferase in Cholesterol Absorption and Its Inhibition by 57-118 in the Rabbit, *J. Lipid Res. 24*, 1127–1134.
15. Clark, S.B., and Tercyak, A.M. (1984) Reduced Cholesterol Transmucosal Transport in Rats with Inhibited Mucosal Acyl CoA:Cholesterol Acyltransferase and Normal Pancreatic Function, *J. Lipid Res. 25*, 148–159.
16. Williams, R.J., McCarthy, A.D., and Sutherland, C.D. (1989) Esterification and Absorption of Cholesterol: *In Vitro* and *in Vivo* Observations in the Rat, *Biochim. Biophys. Acta 1003*, 213–216.
17. Ishida, S., Sato, A., Iizuka, Y., Sawasaki, Y., Aizawa, A., and Kamei, T. (1988), Effects of MK-733, an Inhibitor of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, on Absorption and Excretion of [^{3}H]Cholesterol in Rabbits, *Biochim. Biophys. Acta 963*, 35–41.
18. Ishida, F., Sato, A., Iizuka, Y., Kitani, K., Sawasaki, Y., and Kamei, T. (1989) Effects of MK-733 (Simvastatin), an Inhibitor of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase, on Intestinal Acylcoenzyme A:Cholesterol Acyltransferase Activity in Rabbits, *Biochim. Biophys. Acta 1004*, 117–123.
19. Kam, N.T.P., Albright, E., Mathur, S.N., and Field, F.J. (1989) Inhibition of Acyl CoenzymeA:Cholesterol Acyltransferase activity in CaCo-2 Cells Results in Intracellular Triglyceride Accumulation, *J. Lipid Res. 30*, 371–377.
20. Kahn, B., Wilcox, H.G., and Heimberg, M. (1989), Cholesterol Is Required for Secretion of Very-Low-Density Lipoprotein by Rat Liver, *Biochem. J. 259*, 807–816.
21. Kam, N.T.P., Albright, E., Mathur, S.N., and Field, F.J. (1990)Effect of Lovastatin on Acyl-CoA:Cholesterol O-Acyltransferase (ACAT) Activity and the Basolateral-Membrane Secretion of Newly Synthesized Lipids by CaCo-2 Cells, *Biochem. J. 272*, 427–433.
22. Reimann, F.M., Herold, G., Grosshans, I., Rogler, G., Fellermann, K., and Stange, E.F. (1992) Regulation of Cholesterol Metabolism and Low-Density Lipoprotein Binding in Human Intestinal CaCo-2 Cells, *Digestion 51*, 10–17.
23. Kasim, S.E., LeBoeuf, R.C., Khilnani, S., Tallapaka, L., Dayananda, D., and Jen, K-L.C. (1992) Mechanisms of Triglyceride-Lowering Effect of an HMG-CoA Reductase Inhibitor in a Hypertriglyceridemic Animal Model, the Zucker Obese Rat, *J. Lipid Res. 33*, 1–7.
24. Reinher, E., Rudling, M., Stahlberg, D., Berglund, L., Ewerth, S., Bjorkhem, I., Einarsson, K., and Angelin, B. (1990) Influence of Pravastatin, a Specific Inhibitor of HMG-CoA Reductase, on Hepatic Metabolism of Cholesterol, *N. Eng. J. Med. 323*, 224–228.
25. Owens, D., Collins, P., Johnson, A., Tighe, O., Robinson, K., and Tomkin, G.H. (1991) Hypercholesterolaemia: Simvastatin and Pravastatin Alter Cholesterol Metabolism by Different Mechanisms, *Biochim. Biophys. Acta 1082*, 303–309.
26. Owens, D., Stinson, J., Collins, P., Johnson, A., and Tomkin, G.H (1991) Improvement in the Regulation of Cellular Cholesterologenesis in Diabetes: the Effect of Reduction in Serum Cholesterol by Simvastatin, *Diabetic Med. 8*, 151–156.
27. Ishida, F., Sato, A., Iizuka, Y., and Kamei, T. (1989) Inhibition of Acyl Coenzyme A:Cholesterol Acyltransferase by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase, *Chem. Pharm. Bull. 37*, 1635–1636.
28. Goldfarb, S., and Pitot, H.C. (1972) Stimulatory Effect of Dietary Lipid and Cholestyramine on Hepatic HMG-CoA Reductase, *J. Lipid Res. 13*, 797–801.
29. Hardgrave, J.E., Heller, R.A., Herrera, M.G., and Scallen, T.J. (1979) Immunotitration of HMG-CoA Reductase in Various Physiological States, *Proc. Natl. Acad. Sci. USA. 76*, 3834–3838.
30. Spady, D.K., Turley, S.D., and Dietschy, J.M. (1985) Rates of Low Density Lipoprotein Uptake and Cholesterol Synthesis are Regulated Independently in the Liver, *J. Lipid Res. 26*, 465–472.
31. Goldstein, J.L., and Brown, M.S. (1984) Progress in Understanding the LDL Receptor and HMG-CoA Reductase, Two Membrane Proteins That Regulate Plasma Cholesterol, *J. Lipid Res. 25*, 1450–1461.
32. Tanaka, R.D., Edwards, P.A., Lan, S-F., Knoppel, E.M., and Fogelman, A.M. (1982) The Effect of Cholestyramine and Mevinolin on the Cholesterol Cycle of Rat Hepatic 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase, *J. Lipid Res. 23*, 1026–1031.
33. Bilhartz, L.E., Spady, D.K., and Dietschy, J.M. (1989) Inappropriate Hepatic Cholesterol Synthesis Expands the Cellular Pool of Sterol Available for Recruitment by Bile Acids in the Rat, *J. Clin. Invest. 84*, 1181–1187.
34. Shand, J.H., and West, D.W. (1992) Characterization of a Cytosolic Protein in Rat Liver Inhibiting Neutral Cholesteryl Ester Hydrolase, *Lipids 27*, 406–412.
35. Shand, J.H., and West, D.W. (1991) Acyl-CoA:Cholesterol Acyltransferase Activity in the Rat Mammary Gland: Variation During Pregnancy and Lactation, *Lipids 26*, 150–154.
36. Shand, J.H., and West, D.W. (1991) Effects of an Antiserum to Rat Growth Hormone and Bromocriptine on Cholesterol-metabolizing Enzymes in the Lactating Rat Mammary Gland, *J. Endocrinol 128*, 287–295.
37. Shand, J.H., West, D.W., McCartney, R.J., Noble, R.C., and Speake, B.K. (1993) The Esterification of Cholesterol in the Yolk Sac Membrane of the Chick Embryo, *Lipids 28*, 621–625.
38. Batzri, S., and Korn, E.D. (1973) Single Bilayer Liposomes Prepared Without Sonication, *Biochim. Biophys. Acta 298*, 1015–1019.
39. Folch, J., Lees, M., and Sloane-Stanley, G.N. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
40. Gamble, W., Vaughan, M., Kruth, H.S., and Avigan, J. (1978) Procedure for Determination of Free and Total Cholesterol in Micro- or Nanogram Amounts for Studies With Cultured Cells, *J. Lipid Res. 19*, 1068–1070.
41. McGowan, M.W., Artiss, J.D., Strandbergh, D.R., and Zak, B. (1983) Enzymic Colorimetry of Lecithin and Sphingomyelin in Aqueous Solution, *Clin. Chem. 29*, 538–542.
42. Bradford, M.M. (1976) A Rapid and Sensitive Method for the

Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72,* 248–254.
43. Labarca, C., and Paigen, K. (1980) A Simple, Rapid and Sensitive DNA Assay Procedure, *Anal. Biochem. 102,* 344–352.
44. Ghosh, S., Kounnas, M.Z., and Grogan, W.M. (1990) Separation and Differential Activation of Rat Liver Cytosolic Cholesteryl Ester Hydrolase, Trigylceride Lipase and Retinyl Palmitate Hydrolase by Cholestyramine and Protein Kinases, *Lipids 25,* 221–225.
45. Shand, J.H., and West, D.W. (1994) The Effects of Clofibrate and Bezafibrate on Cholesterol Metabolism in the Liver of the Male Rat, *Lipids 29,* 747–752
46. Shand, J.H., and West, D.W. (1995) The Effects of Probucol and Clofibrate Alone and in Combination on Hepatic Cholesterol Metabolism in the Male Rat, *Biochim. Biophys. Acta 1255,* 123–130.
47. Edwards, P.A., Lan, S-F., and Fogelman, A.M. (1983) Alterations in the Rates of Synthesis and Degradation of Rat Liver 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Produced by Cholestyramine and Mevinolin, *J. Biol. Chem. 258,* 10219–10222.
48. Erickson, S.K., Shrewsbury, M.A., Brooks, C., and Meyer, D.J. (1980) Rat Liver Acyl-Coenzyme A:Cholesterol Acyltransferase: Its Regulation *in Vivo* and Some of Its Properties *in Vitro, J. Lipid Res. 21,* 930–941.
49. Turley, S.D., and Dietschy, J.M. (1984) Modulation of the Stimulatory Effect of Pregnenolone-16α-carbonitrile on Biliary Cholesterol Output in the Rat by Manipulation of the Rate of Hepatic Cholesterol Synthesis, *Gastroenterology 87,* 284–292.
50. Yamauchi, S., Linscheer, W.G., and Beach, D.H. (1991) Increase in Serum and Bile Cholesterol and HMG-CoA Reductase by Lovastatin in Rats, *Am J. Physiol. 23,* G625–G630.
51. Nervi, F., Bronfman, M., Allalon, W., Depiereux, E., and Del Pozo, R. (1984) Regulation of Biliary Cholesterol Secretion in the Rat, *J. Clin. Invest. 74,* 2226–2237.
52. Stone, B.G., Erickson, S.K., Craig, W.Y., and Cooper, A.D. (1985) Regulation of Rat Biliary Cholesterol Secretion by Agents That Alter Intrahepatic Cholesterol Metabolism, *J. Clin. Invest. 76,* 1773–1781.
53. Suckling, K.E., Strange, E.F., and Dietschy, J.M. (1983) Dual Modulation of Hepatic and Intestinal Acyl Coenzyme A:Cholesterol Acyltransferase Activity by (De-)Phosphorylation and Substrate Supply *in Vitro, FEBS Lett. 151,* 111–116.
54. Bell, F.P., Gammill, R.B., and St. John, L.C. (1992) U-73482: A Novel ACAT Inhibitor That Elevates HDL-Cholesterol, Lowers Plasma Triglyceride anf Facilitates Hepatic Cholesterol Mobilization in the Rat, *Atherosclerosis 92,* 115–122.
55. Mitchell, A., Fidge, N., and Griffiths, P. (1993) The Effect of the HMG-CoA Reductase Inhibitor Simvastatin and of Cholestyramine on Hepatic Apolipoprotein mRNA levels in the Rat, *Biochim. Biophys. Acta 1167,* 9–14.
56. Carr, T.P., Parks, J.S., and Rudel, L.L. (1992) Hepatic ACAT Activity in African Green Monkeys Is Highly Correlated to Plasma LDL Cholesteryl Ester Enrichment and Coronary Artery Atherosclerosis, *Arteriosclerosis 12,* 1274–1283.
57. Sviridov, D.D., Pavlov, M.Y., Safonova, I.G., Repin, V.S., and Smirnov, V.N. (1990) Inhibition of Cholesterol Synthesis and Esterification Regulates High Density Lipoprotein Interaction with Isolated Epithelial Cells of Human Intestine, *J. Lipid Res. 31,* 1821–1830.
58. Benhiza, F., Lagrange, D., Malewiak, M-I., and Griglio, S. (1994) *In Vivo* Regulation of Hepatic Lipase Activity and mRNA Levels by Diets Which Modify Cholesterol Influx to the Liver, *Biochim. Biophys. Acta 1211,* 181–188.
59. Vyas, K.P., Kari, P.H., Prakash, S.R., and Duggan, D.E. (1990) Biotransformation of Lovastatin II. *In Vivo* Metabolism by Rat and Mouse Liver Microsomes and Involvement of Cytochrome P-450 in Dehydrogenation of Lovastatin, *Drug Metab. Dispos. 18,* 218–222.
60. Vickers, S., Duncan, C.A., Vyas, K.P., Kari, P.H., Arison, B., Prakash, S.R., Ramjit, H.G., Pitzenberger, S.M., Stokker, G., and Duggan, D.E. (1990) *In Vitro* and *In vivo* Biotransformation of Simvastatin, an Inhibitor of HMG CoA Reductase, *Drug Metab. Dispos. 18,* 476–483.
61. Parker, R.A., Clark, R.W., Sit, S-Y., Lanier, T.L., Grosso, R.A., and Wright, J.J.K. (1990) Selective Inhibition of Cholesterol Synthesis in Liver versus Extrahepatic Tissues by HMG-CoA Reductase Inhibitors, *J. Lipid Res. 31,* 1271–1282.

[Received January 24, 1995, and in final revised form May 24, 1995; Revision accepted June 27, 1995]

Proteolytic Susceptibility of Platelet Low Density Lipoprotein Receptor[1]

Javier Pedreño* and Rosa Fernández

Department of Biochemistry, Hospital Universitario Son Dureta, Palma de Mallorca, Spain

ABSTRACT: In order to further characterize low density lipoprotein (LDL)-platelet interaction, we investigated the effect of protease pretreatment of human platelets on the subsequent binding of iodinated LDL (^{125}I-LDL). Our results showed that the platelet LDL receptor had a proteolytic susceptibility different from that of both classical LDL receptors and the fibrinogen receptor. Platelet pretreatment with chymotrypsin, trypsin, and pronase (at 50 μg/mL) had no effect on ^{125}I-LDL binding, whereas fibroblast ^{125}I-LDL binding was markedly reduced. Mild proteolytic digestion, however (up to 1 mg/mL), was helpful in characterizing the platelet LDL receptor. Scatchard analysis showed that chymotrypsin did not modify LDL binding characteristics, whereas trypsin and pronase altered maximal number of binding sites (B_{max}) without variation in dissociation constant. Trypsin increased B_{max} approximately twofold (2156 ± 327 binding sites on control platelets vs. 5246 ± 296 on treated platelets, $P < 0.001$, mean ± SEM, n = 5), but pronase decreased B_{max} about 50% (2017 ± 275 control vs. 1153 ± 195 treated, $P < 0.001$). A minimum of 30 min preincubation was required to detect significant effects, and apparent equilibrium was reached by 60 min. Maximal increase in platelet LDL binding sites induced by trypsin was observed at a protein concentration of 1 mg/mL at 37°C, whereas at 4°C no effect was found. In contrast, maximal pronase-inhibitory effect also was observed at 37°C but at higher protein concentration (10 mg/mL). Aprotinin, phenylmethylsulfonylfluoride, and soybean trypsin inhibitor were capable of fully blocking both the stimulation and the inhibition of platelet LDL binding induced by trypsin and pronase, respectively. Platelet pretreatment with both chymotrypsin and pronase (0.5 mg/mL) activated fibrinogen binding sites to a similar extent as ADP (100 μM). Furthermore, LDL (at a protein concentration of 0.3 mg/mL) increased by 81 ± 6% the binding of fibrinogen to both protease- and ADP-stimulated platelets, but was unable to activate fibrinogen binding sites in unstimulated platelets. Overall, the results suggest that platelet LDL receptor presents a different proteolytic susceptibility in comparison with both "classical" LDL receptor and fibrinogen receptor.

Lipids 30, 927–933 (1995).

[1]Portions of this work were presented at the 17th Meeting of the European Lipoprotein Club, Tutzing, Germany, September 12–15, 1994.

*To whom correspondence should be addressed at: Department of Biochemistry, Hospital Universitario Son Dureta, Andrea Doria 55, Palma de Mallorca 07014, Spain.

Abbreviations: B_{max}, maximal number of binding sites; GP, glycoprotein; ^{125}I-LDL, iodinated low density lipoprotein; K_D, dissociation constant; LDL, low density lipoprotein; PMSF, phenylmethylsulfonylfluoride; STI, soybean trypsin inhibitor.

It is well known that proteolytic enzymes are implicated in a multitude of important physiological processes, including the functional activation or inactivation of proteins (1). Proteolytic enzymes have been used to characterize platelet receptors, and several studies have shown that a protease-specific mechanism is involved in their proteolytic susceptibility (2–7). Pretreating platelets with either chymotrypsin or pronase can activate the inducible fibrinogen receptor *via* proteolysis of glycoprotein (GP) IIB (2,3), increasing the binding of protein C (4), or causing the inhibition of U46619 binding to thromboxane receptors (5). In contrast, pretreating platelets with trypsin induces the functional activation of thrombin receptors (6) and inhibits the binding of α_2-adrenergic agents (7). However, data on the effect of proteases on the platelet low density lipoprotein (LDL) receptor have not yet been published. Several characteristics indicate that this lipoprotein receptor could be different from the "classical" LDL receptor of nucleated cells (8). A 140 kDa LDL-binding protein has been purified (9,10) from platelet membranes, suggesting that it could be related to the fibrinogen receptor (10), but several differences have been described between fibrinogen and LDL binding characteristics (8). Previous studies from our laboratory also have confirmed differences between platelet and classical LDL receptors (11), and that both native and oxidized LDL particles are recognized with the same apparent affinity by this new lipoprotein receptor (12). In order to further characterize platelet LDL receptors, we investigated their susceptibility to proteolytic digestion and the effect of platelet protease pretreatment on the LDL-platelet interaction.

EXPERIMENTAL PROCEDURES

Materials. α-Chymotrypsin, sucrose, and EDTA were purchased from E. Merck (Darmstadt, Germany); Iodo-Gen, trypsin inhibitor (type I-S: from soybean), pronase, aprotinin, and phenylmethylsulfonylfluoride (PMSF) were supplied by Sigma Chemical Co. (St. Louis, MO); fibrinogen was from Kabi (Stockholm, Sweden); TPCK-trypsin was from Biochrom KG (Berlin, Germany); and Na^{125}I was purchased from New England Nuclear (Boston, MA).

Isolation of lipoproteins and fibrinogen. Human LDL (d = 1.019–1.063 g/mL) were obtained from pooled sera of nor-

Copyright © 1995 by AOCS Press

mocholesterolemic volunteers and isolated by sequential ultracentrifugation (13) in a T 2060 Centrikon ultracentrifuge (Kontron Instruments, Milan, Italy). The protein content was determined by the procedure of Bradford (14) using bovine serum albumin as the standard. The apoprotein composition of LDL and its specific mobility were routinely assessed by the procedure previously reported (11). Isolated LDL was dialyzed against buffer A (150 mM NaCl, 20 mM Tris, and 0.3 mM EDTA; pH 7.4) and filtered through a Millipore (Barcelona, Spain) filter (0.22 μm) before use. Human fibrinogen was purified by chromatography on gelatine-Sepharose 4B (Pharmacia Biotech Europe GmbH, Barcelona, Spain). The clottability of this fibrinogen was between 93 to 96%. As judged by SDS–PAGE after reduction with 5% (vol/vol) β-mercaptoethanol, no contaminating protein was detectable and a typical distribution of Aα, Bβ, and ψ chains and absence of any degradation products was revealed. The protein migrated in nonreducing conditions as a single band on SDS–PAGE with an estimated M_r of 330,000.

Iodination of proteins. Both LDL and fibrinogen were labeled with ^{125}I by the Iodo-Gen method (15) and dialyzed as indicated for unlabeled lipoprotein. The final specific radioactivity of iodinated LDL (^{125}I-LDL) was 2.0 ± 0.1 Bq/ng protein. The purity of ^{125}I-LDL was assessed by gel filtration in a fast protein liquid chromatography device (Pharmacia LKB, Uppsala, Sweden) as previously described (11). The specific radioactivity of ^{125}I-fibrinogen was of 3.5 ± 1.2 Bq/ng of protein. The purity of labeled fibrinogen was evaluated by both SDS–PAGE and by its clottability. All labeled proteins were used immediately.

Platelets. Human platelets were isolated from freshly drawn citrated (sodium citrate 3.8%) blood of healthy volunteers. Platelet-rich plasma was obtained by centrifugation at 300 × *g* for 10 min at room temperature. The platelet pellet was sedimented at 900 × *g* for 6 min at room temperature and then resuspended and adjusted to give a platelet count of 10^{12} platelets per liter in a volume of incubation buffer (0.15 M NaCl, 20 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 mM EDTA; pH 7.45) for LDL binding assays, and in a volume of Tyrode's buffer (0.14 M NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.5 mM glucose, 10 mM HEPES, pH 7.45) for fibrinogen binding assays.

Pretreatment of washed human platelets with proteolytic enzymes. Suspensions of washed platelets (0.4 mL) were preincubated with proteases (up to 1 mg/mL) in the absence and presence of different protease inhibitors [PMSF 0.5 mM = 10 μL in ethanol; aprotinin or soybean trypsin inhibitor (STI), 5 μM in buffer] for 1 h at 37, 22, and 4°C, following the procedure previously described (2). Control (untreated) platelet suspensions also were incubated under the same conditions. The effect of ethanol on both platelet LDL and fibrinogen binding was assessed by control experiments without PMSF. Finally, in order to inhibit proteolysis at the end of the incubation period in the samples without protease inhibitors, PMSF (0.5 mM = 10 μL in ethanol) and aprotinin (5 μM) were added to the incubation mixture. Platelets were then recovered by centrifugation at 1,600 × *g* for 15 min at room temperature and the final volume adjusted to give a platelet count of 10^{12} platelets per liter with incubation buffer (for LDL binding) or Tyrode's buffer (for fibrinogen binding), both containing bovine serum albumin (5 g/L).

Ligand-binding assays. ^{125}I-LDL binding (0.05 mg of protein/mL) to control and protease-pretreated platelets was determined following the procedure previously described (11,12). Nonspecific binding was defined as binding not displaced by a 20-fold excess of unlabeled LDL. After incubation for 25 min, aliquots (0.2 mL) were removed, layered onto 0.8 mL of 20% sucrose in incubation buffer in Eppendorf tubes, and centrifuged at room temperature for 3 min at 16,000 × *g*. The platelet pellet was then recovered by cutting off the tips of the tubes and counted for radioactivity.

Platelet ^{125}I-fibrinogen binding (100 nM) was determined following the procedure previously reported (2). Washed human platelets were incubated with labeled fibrinogen for 5 min at room temperature, and nonspecific binding was obtained in the presence of a 40-fold excess of unlabeled fibrinogen. Finally, specific high-affinity platelet fibrinogen binding was determined as described above for LDL.

Cultured human skin fibroblasts were grown and maintained in a humidified 5% CO_2 incubator at 37°C in 75-cm^2 flasks, and high-affinity binding was measured following the procedure previously described (16). Briefly, fibroblasts were seeded at 5 × 10^4 in 35-mm culture wells and grown in minimal essential medium containing 10% (vol/vol) fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 24 mmol/L $NaHCO_3$, 1 mmol/L pyruvate, and 2 mmol/L L-glutamine. The growth medium was replaced every three days. At confluence, the medium was removed, and the cells were incubated in lipoprotein-deficient serum [2 mL fresh medium containing 5% (vol/vol) lipoprotein-deficient serum instead of fetal calf serum) for 24 h prior to the experiment. The effect of proteases on ^{125}I-LDL binding to human fibroblast was determined following the procedure previously reported by Kovanen *et al.* (17). Calculation of bound lipoprotein was based on the specific activity of labeled LDL, and the results expressed as nanograms of protein bound per 10^8 platelets and as nanograms of protein bound per milligram of total cell protein for fibroblasts. Specific binding was evaluated mathematically by Scatchard analysis (18) to determine the number of binding sites and the dissociation constant by using the KINETIC/EBDA/LIGAND program (19).

Statistical analysis. Results are expressed as mean ± SEM. Differences between means were compared by Student's *t*-test, with $P < 0.05$ considered significant.

RESULTS

Table 1 shows the effect of pretreatment with proteases at varying concentrations on platelet and fibroblast LDL receptors. Under our experimental conditions, platelet pretreatment up to 50 μg/mL with chymotrypsin, pronase, or trypsin had no effect on ^{125}I-LDL binding, and similar values of bound

TABLE 1
Effect of Treatment with Chymotrypsin, Pronase, or Trypsin on Both Platelet and Fibroblast LDL Binding[a]

	^{125}I-LDL bound (ng)	
	Platelets	Fibroblasts
None	175 ± 12	340 ± 29
Chymotrypsin		
1 mg/mL	169 ± 8	100 ± 8
50 mg/mL	158 ± 19	98 ± 12
Trypsin		
1 mg/mL	183 ± 12	88 ± 11
50 mg/mL	170 ± 8	84 ± 8
Pronase		
1 mg/mL	191 ± 13	101 ± 12
50 mg/mL	169 ± 7	78 ± 13

[a]Iodinated low density lipoprotein (^{125}I-LDL) binding (0.05 mg/mL) to control and pretreated platelets and fibroblasts was determined as described in the Experimental Procedures section. Specific high-affinity binding is expressed as nanograms of ^{125}I-LDL bound per milligram of total cell protein for fibroblast and as nanograms of ^{125}I-LDL bound per 10^8 platelets. Results are mean ± SEM of three independent experiments.

LDL were obtained in control and protease-treated platelets. In contrast, at the same concentration, ^{125}I-LDL binding to human fibroblasts was reduced to 70 ± 10% of control (mean ± SEM, n = 3). However, proteolytic digestion (up to 1 mg/mL) was useful to characterize the platelet LDL receptor (Fig. 1). In these experimental conditions, chymotrypsin pretreatment did not exert any significant effect on ^{125}I-LDL binding; however, in a dose-dependent manner, both trypsin and pronase pretreatment increased and decreased the platelet ^{125}I-LDL binding to 140 ± 20 and 50% ± 11% of control (mean ± SEM, n = 7), respectively. Maximal effects of trypsin and pronase were observed at protein concentrations of 1 and 10 mg/mL, respectively.

Figure 2 shows saturation curves of ^{125}I-LDL binding to protease-treated and control platelets. Scatchard analysis of

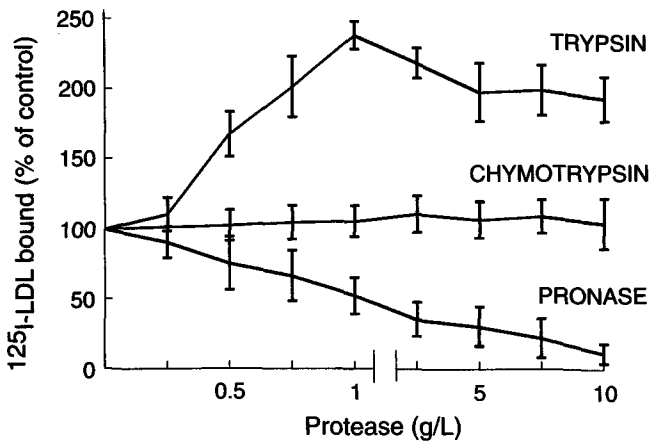


FIG. 1. Effect of proteolytic digestion on platelet low density lipoprotein (LDL) receptor. ^{125}I-LDL binding (0.05 mg/mL) to protease-pretreated platelets was determined as described in the Experimental Procedures section. Results are mean ± SEM of seven independent experiments performed in duplicate.

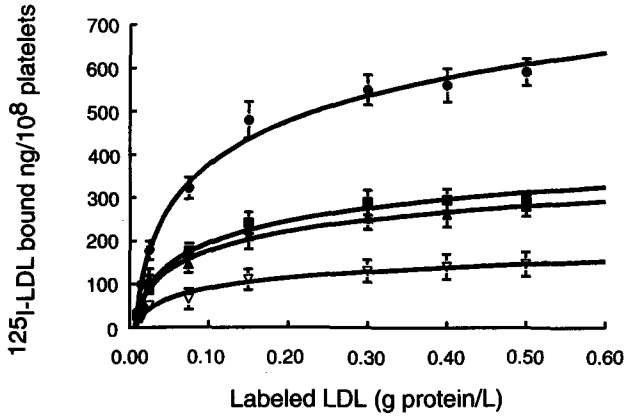


FIG. 2. Saturation curves of ^{125}I-LDL binding to treated pronase (▽), chymotrypsin (▲), trypsin (●), and control (■) platelets. Experimental procedures were carried out as described in the Experimental Procedures section. Platelets (0.4 mL) were preincubated with trypsin, chymotrypsin, and pronase (at a protein concentration of 1 mg/mL) for 1 h at 37°C. Control-unpretreated platelets were also incubated under the same conditions. Afterwards, platelets were recovered by centrifugation and specifically bound ^{125}I-LDL was determined as described in the Experimental Procedures section. Each point is mean ± SEM of five duplicate independent experiments. See Figure 1 for abbreviations.

binding data (Fig. 3) obtained with chymotrypsin-treated platelets showed binding characteristics similar to those of untreated platelets [maximal number of binding sites (B_{max}) = 1967 ± 256 binding sites with a dissociation constant (K_D) of 28 ± 9 nM, and B_{max} = 2119 ± 318 binding sites with a K_D of 31 ± 12 nM, respectively; mean ± SEM, n = 5]. Nevertheless, trypsin-treated platelets showed functional alterations in binding characteristics; B_{max} increased approximately twofold (2156 ± 327 binding sites vs. 5246 ± 296 binding sites, $P < 0.001$, mean ± SEM, n = 5), whereas K_D remained unaltered (31.4 ± 7.8 nM vs. 41.2 ± 10.1 nM, mean ± SEM, n = 5). Binding data obtained with pronase-pretreated platelets showed the opposite effects on B_{max} values, which decreased about 50% (2017 ± 275 binding sites vs. 1153 ±

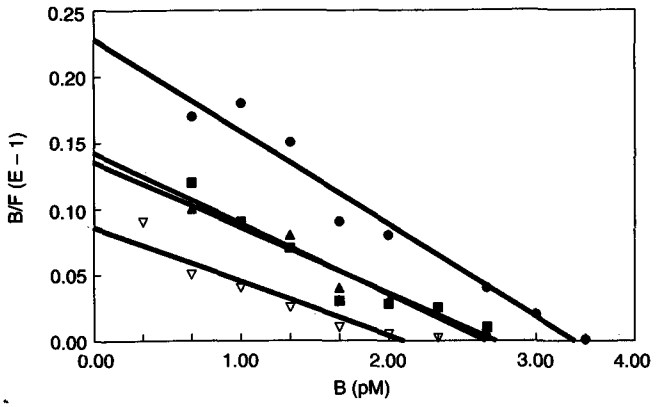


FIG. 3. Scatchard plots of the saturation curves of ^{125}I-LDL binding to treated pronase (▽), chymotrypsin (▲), trypsin (●), and control (■) platelets. See Figure 1 for abbreviation.

195 binding sites), but again with no change in K_D values were found (36 ± 9 nM vs. 33 ± 6 nM, mean ± SEM, n = 5).

The stimulatory effect on ^{125}I-LDL binding induced by trypsin was temperature-dependent. On preincubating at 22 and 37°C, trypsin increased the binding of LDL about 60 ± 15 and 140 ± 20% of control, respectively, whereas at 4°C trypsin was unable to modify platelet LDL binding (Fig. 4B). A minimal preincubation period of 30 min was necessary to observe significant effects, maximal effect being reached after incubation for 60 min at 37°C (Fig. 4A). These results would indicate that both the temperature and time effects relate to protease activity and have nothing to do with LDL-binding isotherms.

Specificity of the proteolytic effect was assessed using trypsin inhibitors (Table 2). Both the trypsin stimulatory and pronase inhibitory effects were fully blocked by 0.5 mM PMSF and 5 μM aprotinin or 5 μM STI. Protease inhibitors at varying protein concentrations were unable to displace the ^{125}I-LDL bound to protease-treated platelets, and inhibition required the addition of trypsin inhibitors prior to the proteolytic enzymes, suggesting that this inhibition was not due to displacement of ^{125}I-LDL bound to its receptor.

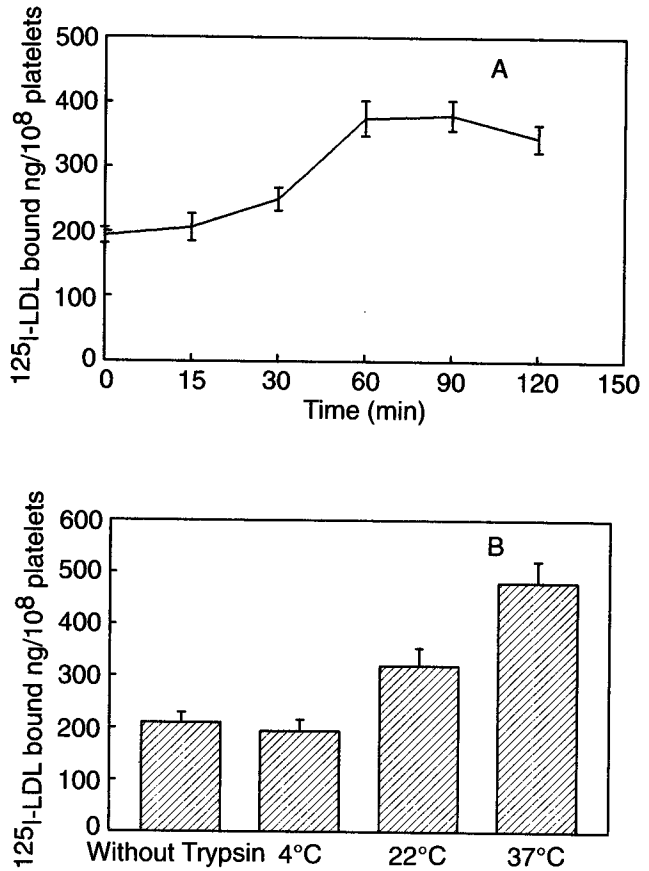


FIG. 4. Effect of time (A) and temperature (B) on the effect of trypsin platelet (1 mg/mL) pretreatment of platelets on subsequent binding of ^{125}I-LDL (0.05 g/L). Results represent mean ± SEM of three independent experiments performed in duplicate. See Figure 1 for abbreviation.

TABLE 2
Effect of Trypsin Inhibitors on the Stimulatory Effect Induced by Trypsin[a]

Addition	Trypsin (mg/mL) None	0.1	0.5	1.0	2.0
None	98 ± 6	110 ± 9	156 ± 12	228 ± 13	211 ± 14
PMSF and aprotinin	96 ± 8	99 ± 10	102 ± 9	98 ± 7	104 ± 10
STI	99 ± 12	104 ± 9	102 ± 11	108 ± 9	99 ± 11

[a]Platelets were preincubated with varying concentrations of trypsin for 1 h at 37°C in the presence and absence of phenylmethylsulfonylfluoride (PMSF) (0.5 mM), aprotinin (5 μM), and soybean trypsin inhibitor (STI) (5 μM). Specifically bound ^{125}I-LDL (ng of protein/10^8 platelets) was measured as described in the Experimental Procedures section. Values are mean ± SEM of three independent experiments. Similar results were obtained for inhibitory effect of pronase.

The effect of chymotrypsin pretreatment of platelets on ^{125}I-fibrinogen (100 nM) binding to unstimulated platelets in the presence and absence of LDL is shown in Figure 5. Figure 5 also shows the effect of LDL on ^{125}I-fibrinogen binding both to unstimulated and ADP-stimulated platelets. Under our experimental conditions, both platelet chymotrypsin (0.5 mg/mL) and pronase (results not shown) activated fibrinogen binding sites in a similar manner to ADP (16 ± 3 ng of ^{125}I-fibrinogen bound/10^8 platelets vs. 12 ± 3 ng of ^{125}I-fibrinogen bound/10^8 platelets). Furthermore, LDL at a protein concentration of 0.3 mg/mL increased ^{125}I-fibrinogen binding to both chymotrypsin-treated and ADP-stimulated platelets (29 ± 2 ng of ^{125}I-fibrinogen bound/10^8 platelets vs. 24 ± 2 ng of ^{125}I-fibrinogen bound/10^8 platelets, respectively). In contrast, LDL at the same protein concentration was unable to activate fibrinogen receptors in unstimulated platelets. These results suggest that LDL increases fibrinogen binding to proteolytically- and ADP-stimulated platelets, whereas in unstimulated

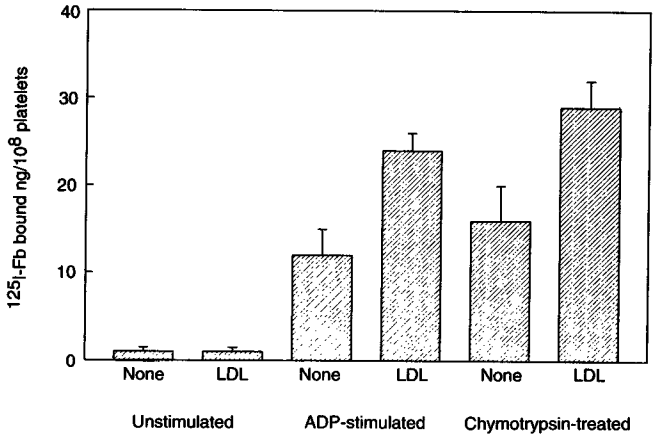


FIG. 5. Effect of LDL (0.3 mg/mL) on ^{125}I-fibrinogen (^{125}I-Fb) binding to chymotrypsin-treated, ADP-stimulated, and unstimulated platelets. Specific high-affinity binding of ^{125}I-fibrinogen (100 nM) in the presence of LDL was determined as indicated in the Experimental Procedures section. Values represent mean ± SEM of four independent experiments performed in triplicate.

platelets this lipoprotein particle is unable to activate GPIIB-IIIA complex.

Finally, it is well known that trypsin at a protein concentration above 1.5 μg/mL induces platelet aggregation (6). Under our experimental conditions, binding of ^{125}I-fibrinogen to trypsin-treated platelets was impracticable both using Tyrode's and incubation buffer, suggesting that a different mechanism *via* GPIIB-IIIA is involved in both LDL and fibrinogen binding to human intact platelets.

DISCUSSION

Plasma cholesterol has been considered as a systemic risk factor for thrombus formation on ruptured atherosclerotic plaques (20). LDL-platelet interactions may explain the increased thrombogenicity described in atheromatous plaques of hypercholesterolemic rabbits (21), the initial step in the modulation of this process being the binding of LDL to a specific platelet receptor (22).

Since the first descriptions of the existence of specific, high-affinity, and saturable binding sites for LDL in platelets, evidence has been presented indicating that these binding sites could be different from the "classical" LDL receptor of nucleated cells (8–12). In fact, Koller (23) first reported that three platelet membrane proteins of 156, 130, and 115 kDa bound LDL specifically. Further studies identified platelet membrane glycoprotein IIB-IIIA complex (the inducible fibrinogen receptor) as responsible for platelet–LDL interactions (10). We have demonstrated that a monoclonal anti-LDL receptor (IgG-C7) was unable to block binding of LDL to control platelets (11). Our most recent findings revealed that the platelet LDL receptor, in contrast to the "classical" LDL receptor of human fibroblasts, does not mediate an endocytotic response and recognizes both native and oxidized LDL particles with the same apparent affinity (12). These data strengthen the hypothesis that platelet LDL receptor differs from "classical" LDL receptor, but on the basis of LDL binding characteristics, several conflicting results concerning the implication of GP IIB-IIIA complex on platelet LDL binding have been presented (8). Nevertheless, GP IV or CD-36, a GP resistant to proteolytic digestion, appears to be involved in the binding of oxidized LDL particles to both monocyte-derived macrophages and platelets, and it has been suggested that this GP could be a new lipoprotein receptor (24,25). Since our previous results found that the platelet LDL receptor binds both oxidized and native LDL particles with the same apparent affinity (12), and implicated a GP resistant to proteolytic digestion in the binding of oxidized LDL, we have attempted to characterize the effect of several proteases on the platelet LDL receptor, and the functional consequences of this effect.

In this study, we have demonstrated that protease treatment, prior to incubation with ^{125}I-LDL, totally abolished fibroblast LDL binding but did not alter platelet LDL binding. In addition, the platelet LDL receptor was resistant to chymotrypsin treatment (up to 10 mg/mL); however, mild pronase treatment (1 mg/mL) was able to reduce platelet LDL binding approximately fifty percent, and more extensive proteolytic digestion (10 mg/mL) caused total inhibition of the binding. Taken together, these data suggest that a different type of cell-surface protein receptor is involved in the binding of LDL to human platelets, and are in agreement with our previous results (11,12).

Treating platelets with chymotrypsin and pronase up to 1 mg/mL produces neither platelet shape change nor aggregation (2). In contrast, 1 μg/mL trypsin induces platelet shape change and aggregation at higher concentrations (26). When we preincubated platelets with trypsin (up to 1 mg/mL) in the presence of 1 mM EDTA, both platelet shape and aggregation were prevented (results not shown), whereas in the absence of EDTA, trypsin caused a complete aggregation. Our results also found increased LDL binding sites when human platelets were treated with trypsin in the presence of EDTA (1 mM). Enhanced lipoprotein binding by platelets after mild trypsin treatment has not been previously reported; however, a stimulatory effect on high density lipoprotein binding to peripheral blood lymphocytes has been described (27). In the present study, our data show that mild trypsin treatment (1 mg/mL) increases platelet LDL binding without changes in dissociation constant and that this stimulatory effect is dependent upon dose, time, and temperature. Moreover, a minimal preincubation period of 30 min is necessary to find significant effects, suggesting that protease effect is caused by proteolysis and not by platelet activation.

Specificity of the effect of proteases on ^{125}I-LDL binding to platelets was assessed in the presence and absence of protease inhibitors. Equimolar concentrations of several protease inhibitors such as STI, aprotinin, and PMSF, led to a total reduction of the proteolytic effect. The effect of protease inhibitors was principally the result of a noncompetitive interaction with the proteases, and cannot be attributed to a competitive inhibitory mechanism.

Finally, it is well known that the GPIIB-IIIA complex is unable to bind fibrinogen unless platelets are activated by agonists such as ADP or proteolytic enzymes, such as chymotrypsin and pronase (2). Conversion of platelet membrane GPIIB and GPIIIA from the latent state to a GPIIB-IIIA complex is a calcium-dependent process. Therefore, the binding of fibrinogen requires this divalent ion and chelating agents cause the dissociation of the complex, fully inhibiting both fibrinogen binding and platelet aggregation (28). It has recently been demonstrated that LDL increases the exposure of fibrinogen receptors instead of inducing inhibition in intact resting platelets (29). The authors postulated that GPIIB-IIIA probably assumes a different conformation in experimental conditions which allow the binding of LDL, and in intact platelets GPIIB-IIIA complex is unable to bind LDL. The results of this study show that chymotrypsin–pretreatment did not alter LDL binding characteristics, whereas it can activate fibrinogen receptors. Moreover, in agreement with van Willigen *et al.* (29), our data demonstrate that activation of fibrinogen binding sites induced by ADP was enhanced in the presence of LDL, and in a similar manner this lipoprotein particle

increased the binding of fibrinogen to protease-treated platelets. In contrast, when platelet aggregation was prevented (incubation buffer containing EDTA), LDL binding was increased by trypsin-pretreatment, whereas fibrinogen binding was unrealizable. All the results indicate that the platelet LDL receptor has a different proteolytic susceptibility compared to both "classical" LDL receptors and fibrinogen receptors, suggesting that the binding of LDL to intact platelets could be related to GP other than GPIIB-IIIA.

A characteristic feature of platelet membrane GP (30,31) is their susceptibility to proteolysis caused by moderate trypsin concentrations (1 mg/mL). GP IV or CD-36, however, has been found to exhibit extensive resistance to proteolytic digestion (32). It has been reported that in both platelets and monocyte-derived macrophages, GP IV-bound oxidized LDL particles with high affinity (24,25). These data, combined with our previous results, suggest that a GP resistant to proteolytic digestion, such as GP IV, could be related to the binding of lipoproteins to intact platelets. The significance of this potential platelet LDL receptor regulation mechanism involving proteolysis needs to be explored in further studies.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Comisión Interministerial de Ciencia y Tecnología (CICYT, 94-0191) and the Spanish Arteriosclerosis Foundation (1991-1).

REFERENCES

1. Neurath, H. (1990) in *Proteolytic Enzymes. A Practical Approach* (Beynon, R.J., and Bond, J.S., eds.) pp. 1–45, IRL Press, Oxford.
2. Niewiarowski, S., Brudzynski, A.Z., Morinelli, T.A., Brudzynski, T.M., and Stewart, G.J. (1981) Exposure of Fibrinogen Receptor on Human Platelets by Proteolytic Enzymes, *J. Biol. Chem. 256*, 917–923.
3. Pidard, D., Frelinger, A.L., Bouillot, C., and Nurden, A.T. (1991) Activation of the Fibrinogen Receptor on Human Platelets Exposed to Alpha Chymotrypsin. Relationship with a Major Proteolytic Cleavage at the Carboxyterminus of the Membrane Glycoprotein IIb Heavy Chain, *Eur. J. Biochem. 200*, 437–443.
4. Fiedel, B.A., Siegel, S.N., Gewurz, H., Simpson, R.M., and Izzi, J.M. (1982) Comparison of the Enzymatic Sensitivities of the Platelet Receptor for Human C-Reactive Protein and Its Functional Relationship to the Platelet IgG Fc Receptor, *Clin. Exp. Immunol. 50*, 215–221.
5. Lukasiewicz, H., Peng, M.L., Morinelli, T.A., Eckardt, A., Kirby, E.P., and Niewiarowski, S. (1989) Separation of Different Receptor-Mediated Effects of a Prostaglandin H2 Analogue (U46619) on Human Platelets by Means of Human Granulocytic Elastase and Chymotrypsin, *Biochem. Pharmacol. 38*, 3213–3218.
6. Brass, L.F., Vassallo, R.R., Belmomte, E., Ahuja, M., Cichowski, K., and Hoxie, J.A. (1992) Structure and Function of the Human Platelet Thrombin Receptor. Studies Using Monoclonal Antibodies Directed Against a Defined Domain Within the Receptor N-Terminus, *J. Biol. Chem. 267*, 13795–13802.
7. Periyasamy, S., and Somani, P. (1986) Effect of Proteases and Phospholipases on [3H] Yohimbine Binding to Human Platelet Membranes, *Biochem. Pharmacol. 35*, 3131–3136.
8. Malle, E., and Satller, W. (1994) Platelets and the Lipoproteins: Native, Modified and Platelet Modified Lipoproteins, *Platelets 5*, 70–83.
9. Hassall, D.G., Desai, K., Owen, J.S., and Bruckorfer, K.R. (1990) Detection of a Protein in Human Platelet Membranes Which Binds Low-Density Lipoproteins, *Platelets 1*, 29–35.
10. Koller, E., Koller, F., and Binder, B. (1989) Purification and Identification of the Lipoprotein-Binding Proteins from Human Blood Platelet Membrane, *J. Biol. Chem. 264*, 12412–12418.
11. Pedreño, J., de Castellarnau, C., Cullaré, C., Gómez, J., Sánchez, J.L., Ordoñez, J., and Gonzalez, F. (1992) LDL Binding Sites on Platelets Differ from the "Classical" Receptor of Nucleated Cells, *Arterioscler. Thromb. 12*, 1353–1362.
12. Pedreño, J., de Castellarnau, C., Cullaré, C., Ortìn, R., Sánchez, J.L., Llopart, R., and González, F. (1994) Platelet LDL Receptor Recognizes with the Same Apparent Affinity Both Oxidized and Native LDL. Evidence That the Receptor-Ligand Complexes Are Not Internalized, *Arterioscler. Thromb. 14*, 401–408.
13. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) The Distribution and Chemical Composition of Ultracentrifugally Separated Lipoproteins in Human Serum, *J. Clin. Inves. 34*, 1345–1353.
14. Bradford, M.A. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye-Binding, *Anal. Biochem. 72*, 248–254.
15. Salacinski, P.R., McLean, C., Sykes, J.E., Clement-Jones, V.V., and Lowry, P.J. (1981) Iodination of Proteins, Glycoproteins, and Peptides Using a Solid-Phase Oxidizing Agent, 1,3,4,6-Tetrachloro-3,6-diphenylglycoluril (Iodogen), *Anal. Biochem. 117*, 136–146.
16. Pedreño, J., Sánchez, J.L., Zambón, D., and Ros, E. (1994) *In vitro* Effect of 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG–CoA) Reductase Inhibitors on Non-Hepatic LDL Receptor Activity: Evidence of Lack of Stimulatory Effect of Pravastatin, *Coron. Artery Dis. 5*, 971–978.
17. Kovanen, P.T., Brown, M.S., and Goldstein, J.L. (1979) Increased Binding of Low Density Lipoprotein to Liver Membranes from Rats Treated with 17 α-Ethinyl Estradiol, *J. Biol. Chem. 254*, 11367–11373.
18. Scatchard, G. (1949) The Attractions of Proteins for Small Molecules and Ions, *Ann. N.Y. Acad. Sci. 51*, 660–672.
19. McPherson, G.A. (1985) Analysis of Radioligand Binding Experiments: A Collection of Computer Programs for the IBM PC, *J. Pharmacol. Methods. 14*, 213–228.
20. Fuster, V., Badimon, L., Badimon, J.J., and Chesebro, J.H. (1992) The Pathogenesis of Coronary Artery Disease and the Acute Coronary Syndromes, *N. Eng. J. Med. 326*, 310–318.
21. Badimon, J.J., Badimon, L., Turitto, V.T., and Fuster, V. (1991) Platelet Deposition at High Shear Rates is Enhanced by High Plasma Cholesterol Levels, *Arterioscler. Thromb. 11*, 395–402.
22. Andrews, H.E., Aitken, J.W., Hassall, D.G., Skinner, V.O., and Bruckdorfer, K.R. (1987) Intracellular Mechanisms in the Activation of Human Platelets by Low-Density Lipoproteins, *Biochem. J. 242*, 550–564.
23. Koller, E. (1986) Lipoprotein-Binding Proteins in the Human Platelet Plasma Membrane, *FEBS Lett. 200*, 97–102.
24. Endemann, G., Stanton, L., Madden, K., Bryant, K., While, R.T., and Protter, A. (1993) CD36 Is a Receptor for Oxidized Low Density Lipoproteins, *J. Biol. Chem. 268*, 1181–11816.
25. Nicholson, A.C., Prieda, S., Pearce, A., and Silverstein, R.L. (1995) Oxidized LDL Binds to CD36 on Human Monocyte-Derived Macrophages and Transfected Cell Lines. Evidence Implicating the Lipid Moiety of the Lipoprotein as the Binding Site, *Arterioscler. Thromb. Vasc. Biol. 15*, 269–275.
26. Finotti, P. (1992) Differential Involvement of Membrane (Na-K) ATPase in Thrombin- and Trypsin-Mediated Platelet Activation, *Thromb. Res. 66*, 669–678.

27. Jürgens, G., Xu, Q., and Huber, L.A. (1989) Promotion of Lymphocyte Growth by High Density Lipoproteins (HDL), *J. Biol. Chem. 264*, 8549–8556.
28. Fitzgerald, L.A., and Phillips, D.R. (1985) Calcium Regulation of the Platelet Membrane Glycoprotein IIb-IIIa Complex, *J. Biol. Chem. 260*, 11366–11373
29. van Willigen, G., Gorter, G., Willem, J., and Akkerman, N. (1994) LDLs Increase the Exposure of Fibrinogen Binding Sites on Platelets and Secretion of Dense Granules, *Arterioscler. Thromb. 14*, 41–46
30. Okumura, T., and Jamieson, G.A. (1976) Platelet Glycocalicin I. Orientation of Glycoproteins of the Human Platelet Surface, *J. Biol. Chem. 251*, 5944–5949.
31. Podolsak, B. (1977) Platelet Membrane Glycoproteins Proteolytic Susceptibility, *Thromb. Haemostasis 37*, 396–406.
32. Tandon, N.N., Lipsky, R.H., Burgess, W.H., and Jamieson, G.A. (1989) Isolation and Characterization of Platelet Glycoprotein IV (CD36), *J. Biol. Chem. 264*, 7570–7575.

[Received March 16, 1995, and in final revised form July 5, 1995; Revision accepted July 31, 1995]

Effects of Randomization of Partially Hydrogenated Corn Oil on Fatty Acid and Cholesterol Absorption, and Tissue Lipid Levels in Rats

Tamiho Koga[a,*], Takako Yamato[a], Ikuo Ikeda[b] and Michihiro Sugano[c]

[a]Department of Food and Nutrition, Nakamura Gakuen University, Jonan-ku, Fukuoka 814-01, [b]Laboratory of Nutrition Chemistry and [c]Laboratory of Food Science, Kyushu University School of Agriculture, Higashi-ku, Fukuoka 812-81, Japan

ABSTRACT: Randomization of partially hydrogenated corn oil containing approximately 45% of *trans* octadecenoic acid only slightly, but not significantly, increased the lymphatic fatty acid absorption in rats. No effect of randomization was observed on cholesterol absorption. When rats were fed these fats at the 8.8% level (with 1.2% safflower oil) for three weeks, the concentrations of serum cholesterol, and serum and liver phospholipid were significantly higher in randomized fat than in control fat, which was composed of 9% high-oleic safflower oil and 1% palm oil. Liver cholesterol tended to be higher in randomized fat. In contrast, nonrandomized fat was not hyperlipidemic compared to control fat. Although the fatty acid composition of liver phospholipids suggested a possible interference of *trans* fatty acid with the metabolism of linoleic acid to arachidonic acid, there was no effect of randomization. In the two hydrogenated fat groups, *trans* octadecenoic acid was incorporated and distributed similarly in adipose tissue triacylglycerol. These observations indicated that randomization of partially hydrogenated fat is not beneficial to various lipid parameters in rats.
Lipids 30, 935–940 (1995).

The nutritional and physiological functions of dietary fat depend on the structure of triacylglycerol, i.e., the positions of fatty acids esterified in the glycerol skeleton. In classical studies on the effect of the difference in the positional distribution of fatty acids in the triacylglycerol molecule, linoleic acid esterified at position-2 of glycerol was more hypocholesterolemic than that at position-3 (1,2). More recently, Kritchevsky (3) reviewed that the atherogenic propensity of dietary peanut oil in rabbits disappeared when it was interesterified. Sugano *et al.* (4) observed in rats a slight but insignificant reduction of the cholesterol-elevating nature of palm oil after randomization. Similar results were also reported for randomized palm oil (5), lard (6), peanut oil (7), and fish oil (7). In the case of a mixture of coconut oil and palm oil, chemical randomization improved the absorption of long-chain saturated fatty acids (8).

Trans fatty acids have been suspected to be hypercholesterolemic and to disturb lipoprotein metabolism (9–12). Since the use of partially hydrogenated vegetable oils is still increasing in various areas of commercial food processing, it is of interest to know whether the randomization could modify possible untoward effects of *trans* fat. Considering the positional specificity of pancreatic lipase toward triacylglycerol and the efficient micellar solubility of 2-monoacylglycerol, a lipase hydrolysis product, the difference in the positional distribution of fatty acid in the triacylglycerol molecule may influence their intestinal absorption and metabolic fates (8,13).

In this context, it is reasonable to understand the effect of randomization of *trans* fat on its intestinal absorption efficiency. In the study reported here, we examined the effect of interesterification of partially hydrogenated corn oil (PHC) containing a high level of *trans* fatty acid, exclusive of *trans* octadecenoic acid on intestinal absorption of fatty acid and cholesterol in lymph-cannulated rats. The effect of positional fatty acid randomization on lipid metabolism was also studied.

MATERIALS AND METHODS

Fat sources. PHC and its randomized product were a gift from Dr. I. Niiya (The Japan Institute of Oils and Fats, Other Foods Inspection, Tokyo, Japan). For randomization, PHC was interesterified in the presence of sodium–methoxide catalyst (0.5 wt%) at 80°C for 30 min. The purified interesterified fat contained more than 98% of triacylglycerol and <2% remained as mono- and diacylglycerols. The composition and distribution of fatty acids of the fat preparations are shown in Table 1. The interesterification did not change the fatty acid composition of triacylglycerol, but changed the fatty acid positional distribution to a more evenly randomized structure as compared to the fatty acid composition of total and the 2-position of triacylglycerol measured after pancreatic lipase hydrolysis (14). In brief, to approximately 10 mg of fat samples was added 1 mL of Tris buffer, pH 8.0, containing 8 mg of porcine pancreatic lipase (Wako Pure Chemicals, Osaka, Japan), and the mixture was vigorously shaken at 40°C for 30 min. Lipids were then extracted with a 2:1 chloroform–methanol mixture, and 2-monoacylglycerol was separated by thin-layer chromatography.

*To whom correspondence should be addressed.

Abbreviation: PHC, partially hydrogenated corn oil.

Copyright © 1995 by AOCS Press

TABLE 1
Fatty Acid Composition of Partially Hydrogenated Corn Oil (PHC) and Randomized Partially Hydrogenated Corn Oil (RPHC)

Dietary fat	Fatty acid (mol %)				
	16:0	18:0	*t*-18:1	*c*-18:1	18:2
PHC					
Total	12.7	6.3	44.5	36.3	0.2
2-Position	0.9	4.6	47.3	46.8	0.4
1,3-Positions[a]	17.1	7.3	43.9	31.6	0.1
RPHC					
Total	11.9	6.7	44.6	36.6	0.2
2-Position	12.2	7.1	40.1	40.3	0.3
1,3-Positions	11.7	6.5	46.8	34.8	0.2

[a]Calculated value.

Fat absorption experiment. Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) weighing 390 g on average were given *ad libitum* a commercial nonpurified diet (Type NMF; Oriental Yeast Co., Tokyo, Japan) and drinking water. Under Nembutal anesthesia, rats were subjected to cannulation of the left thoracic lymphatic channel, and an indwelling catheter was placed in the stomach. A solution containing 139 mM glucose and 85 mM NaCl was infused continuously until the end of lymph collection at a rate of 3 mL/h through the gastric tube (15). After surgery, animals were placed in restraining cages in a warm recovery room and allowed free access to drinking the isosmotic infusion solution. The next morning, after collection of lymph for 2 h as a control period, each animal was administered 3 mL of a test emulsion through the gastric tube, and the lymph was collected for 24 h, beginning at 1000 hours. The 3-mL test emulsion contained 200 mg of sodium taurocholate, 50 mg of fatty acid-free albumin (Miles Scientific, ICN Immuno Biologicals, Lisle, IL), 1 mCi of [^{14}C] cholesterol (60 mCi/mmol; NEN Research Products, Boston, MA), 25 mg of cholesterol, and 200 mg of fats. The emulsions were prepared by sonication prior to administration. The lymph was collected in a tube containing EDTA at 3-h intervals during the first 9 h after administration of a test emulsion and at a single collection 9–24 h after the emulsion.

Feeding experiment. Male Sprague-Dawley rats five-weeks-old (Seiwa Experimental Animals) were housed individually in a temperature (20–22°C) and light (lights on 0600 to 1800 hours) controlled room. The animals were fed AIN-type purified diets (16) *ad libitum* for three weeks after three days of acclimatization. The diet composition is given in Table 2 together with the fatty acid composition of dietary fat. High-oleic safflower oil served as a control fat, and the content of oleic acid was made equal to that of total octadecenoic acids in hydrogenated fats by mixing with palm oil. By this manipulation, the linoleic acid content was also comparable among three dietary fats. Food intake and body weight were recorded every other day. Feces were collected for two days during the end of third week. After three weeks, rats were killed by decapitation without fasting, and blood collected. Tissues were immediately excised, blotted, and weighed.

TABLE 2
Composition of Diets

Ingredient[a]	Dietary group		
	HOS	PHC	RPHC
	(wt%)		
Casein	20	20	20
High-oleic safflower oil (HOS)	9.0	—	—
PHC	—	8.8	—
RPHC	—	—	8.8
Palm oil	1.0	—	—
High-linoleic safflower oil	—	1.2	1.2
Fatty acid	Fatty acid composition (wt%)		
16:0	11.5	11.0	11.3
18:0	6.5	6.5	6.3
t-18:1	—	38.7	38.4
c-18:1	70.3	32.5	33.3
18:2	11.7	10.0	10.0

[a]Other ingredients were vitamin mixture AIN-76, 1.0; mineral mixture AIN-76, 3.5; choline bitartrate, 0.2; cellulose, 5.0; corn starch, 15.0; and sucrose, 45.3. See Table 1 for other abbreviations.

Lipid analyses. Lymph lipids were extracted by the method of Folch *et al.* (17). Pentadecenoic acid (Aldrich, Milwaukee, WI) was added as an internal standard, and the fatty acid composition was measured by gas–liquid chromatography (18). The fatty acid recovery rate was calculated by subtracting the fatty acid amount in the blank lymph (3–6 mg fatty acids/mL) from that in the lymph collected after the administration of a test emulsion. An aliquot of the lymph was transferred to a scintillation vial, and 10 mL of Aquasol II (NEN Research Products) was added. The radioactivity was measured using a liquid scintillation counter (LSC-1200; Aloka, Tokyo, Japan). Serum and liver lipids were extracted (17), and the concentrations of cholesterol, triacylglycerol, and phospholipids were measured (19). Liver phospholipids were separated into their constituents by thin-layer chromatography (20), and the fatty acid composition and phosphorus content (21) were measured. Fecal lipid contents were measured by the method A of Kamer *et al.* (22). The fatty acid composition of fecal lipids was measured on the chloroform-methanol extract.

Statistical analysis. Student's *t*-test (for the absorption study) and Duncan's multiple range test (for the feeding study) (23) were used to determine the statistical difference between groups in absorption and feeding studies, respectively.

RESULTS

Effects of randomization on fatty acid and cholesterol absorption. The lymph flow rate was comparable between randomized and nonrandomized fats (data not shown). As shown in Figure 1, lymphatic absorption of fatty acid was largely unaltered by the randomization of hydrogenated fat administered (Fig. 1A), but it tended to be slightly higher with the randomized fat than in the nonrandomized fat, particularly 9 h after the administration of the fat emulsion. No effect on choles-

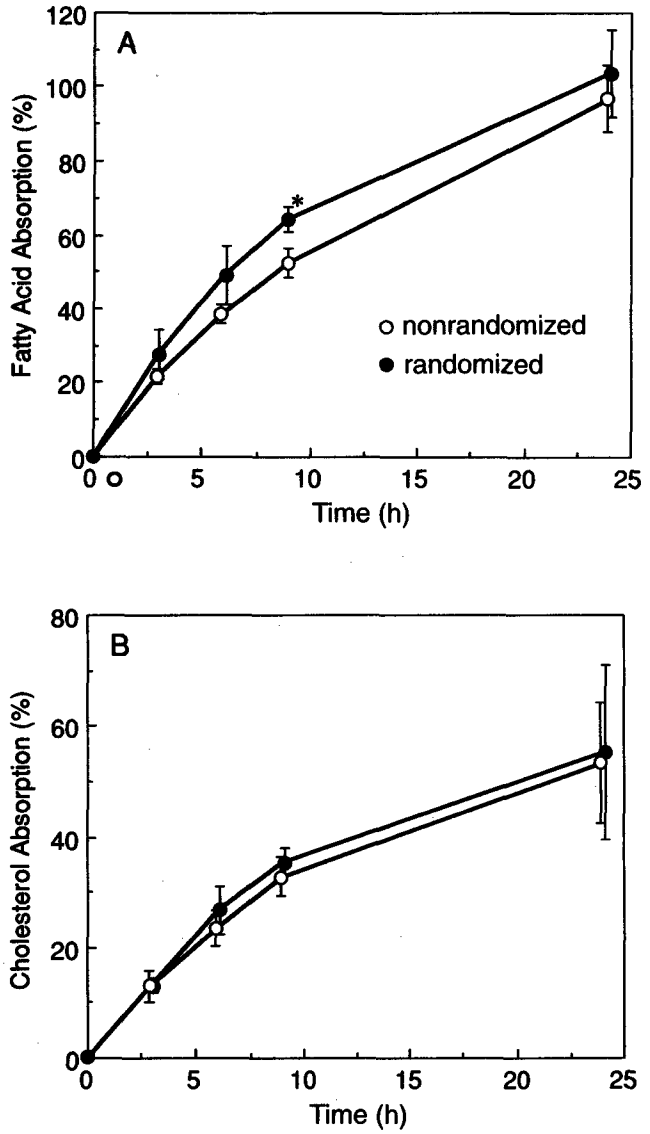


FIG. 1. Effects of randomization of partially hydrogenated corn oil on lymphatic absorption of fatty acid (A) and cholesterol (B). Values are means ± SE of six rats per group. ○, nonrandomized fat; ●, randomized fat. *Significantly different at $P < 0.05$.

terol absorption was observed (Fig. 1B). Lymph collected periodically showed no significant differences in the fatty acid compositions between two types of fats at any time intervals (data not shown).

Effects of randomization on lipid metabolism. In this experiment, we could directly compare the effect of the difference in the geometry of dietary octadecenoic acids (between the control fat and two hydrogenated fats) and the distribution of fatty acid in the triacylglycerol molecule (between two hydrogenated fats) as well.

Food intake and weight gain were comparable among the groups, but the weight of some tissues differed depending on the fat source (Table 3). Thus, relative weight of liver was significantly higher and that of heart significantly lower in rats fed hydrogenated fats than in those fed control fat, high-oleic safflower oil. However, there was no difference in weight between these tissues and two hydrogenated fat groups. Weights of kidney, spleen, and epididymal adipose tissue were all comparable among three groups.

TABLE 3
Effects of Randomization of Partially Hydrogenated Corn Oil on Growth Parameters and Tissue Weights[a]

Parameter	Dietary group		
	HOS	PHC	RPHC
Body weight (g)			
Initial	93 ± 4	91 ± 2	91 ± 2
Gain	161 ± 8	160 ± 5	175 ± 4
Food intake (g/d)	20.3 ± 1.7	18.7 ± 2.0	19.3 ± 2.1
Relative tissue weight (g/100 g body weight)			
Liver	4.76 ± 0.73[b]	5.74 ± 0.51[c]	5.85 ± 0.52[c]
Heart	0.44 ± 0.04[b]	0.38 ± 0.03[c]	0.37 ± 0.03[c]
Kidney	0.96 ± 0.06	1.03 ± 0.09	1.01 ± 0.08
Spleen	0.27 ± 0.05	0.28 ± 0.05	0.27 ± 0.03
Adrenal	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Epididymal adipose tissue	1.59 ± 0.29	1.73 ± 0.12	1.67 ± 0.28

[a]Values are means ± SE of eight rats. See Table 2 for abbreviations.
[b,c]Values with different superscript letters are significantly different at $P < 0.05$.

The concentrations of serum and liver lipids are summarized in Table 4. There was a trend toward increasing serum lipids after randomization, and serum cholesterol and phospholipid were significantly higher in animals receiving randomized fat than in the control fat group, with the nonrandomized fat group being intermediate. The concentrations of liver lipids were again highest in the randomized fat group, and liver phospholipid was significantly higher compared to the control group. However, there was no change in the fatty acid composition of liver phospholipid (data not shown).

The data shown in Table 5 indicate that the fatty acid composition and distribution in adipose tissue triacylglycerol were the same between the two hydrogenated fat groups. Thus, the proportion of *trans*-octadecenoic acid was not different in total and 2-position of triacylglycerol. Table 6 shows that feeding *trans* fats resulted in a significant reduction of arachidonic acid and a significant increase in linoleic acid in

TABLE 4
Effects of Randomization of Partially Hydrogenated Corn Oil on Concentrations of Serum and Liver Lipids[a]

Tissue and lipid	Dietary group		
	HOS	PHC	RPHC
Serum (mg/dl)			
Cholesterol	65.6 ± 5.5[b]	72.6 ± 2.8[b]	86.5 ± 5.0[c]
Triacylglycerol	218 ± 15	224 ± 5	228 ± 17
Phospholipid	142 ± 4[b]	154 ± 2[b,c]	157 ± 6[c]
Liver (mg/g)			
Cholesterol	4.31 ± 0.24	4.30 ± 0.35	5.17 ± 0.33
Triacylglycerol	57.1 ± 7.3	71.9± 5.9	72.4 ± 5.6
Phospholipid	31.9 ± 0.8[b]	33.3 ± 1.2[b,c]	36.4 ± 1.2[c]

[a]Values are means ± SE of 8 rats. See Table 2 for abbreviations.
[b,c]Values with different letters are significantly different at $P < 0.05$.

TABLE 5
Effects of Randomization of Partially Hydrogenated Corn Oil on Fatty Acid Compositions of Epididymal Adipose Tissue Triacylglycerol[a]

	Fatty acid (wt%)						
Dietary group	14:0	16:0	16:1	18:0	*t*-18:1	*c*-18:1	18:2
HOS							
Total	1.5 ± 0.2	22.3 ± 1.3	6.2 ± 0.9	2.2 ± 0.1[b]	—	57.7 ± 1.9[b]	8.1 ± 1.6
2-Position	1.1 ± 0.3	6.1 ± 0.7[d]	4.6 ± 0.8[d]	—	70.3 ± 1.4[d]	14.6 ± 2.7	
PHC							
Total	1.6 ± 0.2	23.9 ± 2.1	6.2 ± 1.0	2.4 ± 0.1[c]	13.6 ± 2.0	40.3 ± 2.4[c]	8.0 ± 0.9
2-Position	1.4 ± 0.2	10.5 ± 0.9[e]	6.4 ± 1.0[e]	1.0 ± 0.1[e]	5.2 ± 0.6	53.3 ± 2.0[e]	16.7 ± 1.9
RPHC							
Total	1.6 ± 0.2	24.2 ± 1.9	6.1 ± 0.9	2.5 ± 2.0[c]	13.7 ± 1.3	40.0 ± 1.2[c]	7.4 ± 0.8
2-Position	1.3 ± 0.2	11.9 ± 0.6[e]	7.0 ± 1.1[e]	1.0 ± 0.2[e]	5.6 ± 0.5	53.0 ± 0.6[e]	15.0 ± 1.3

[a]Values are means ± SE of eight rats. See Table 2 for abbreviations.
[b,c, d,e]Values with different letters are significantly different at $P < 0.05$, respectively.

liver phosphatidylcholine. Linoleic acid was also increased in phosphatidylethanolamine. Thus, the ratio of (20:3n-6 + 20:4n-6)/18:2n-6, an index for linoleic acid desaturation, was significantly lower in the two hydrogenated fat groups. The proportion of docosapentaenoic acid (22:5n-6) decreased significantly on feeding hydrogenated fats. In addition to these changes, there was a significant difference in the proportion of stearic and palmitoleic acids. In liver triacylglycerol, palmitic and palmitoleic acids were significantly higher in rats fed hydrogenated fats than in those fed high-oleic safflower oil.

Rats fed hydrogenated fats excreted more lipids in their feces (0.26 ± 0.04 and 0.22 ± 0.03 g/d for nonrandomized and randomized fats, respectively) than did rats fed high-oleic safflower oil (0.13 ± 0.04 g/d, different from the two hydrogenated fat groups at $P < 0.05$). There was, however, no significant difference in the fecal lipid level between the two hydrogenated fat groups. Feeding hydrogenated fats resulted in a significant reduction of the proportion of palmitic and stearic acids in feces, while increasing *trans* octadecenoic acid (Table 7). The same level of *t*-18:1 was found in rats fed hydrogenated fats. A small amount of *t*-18:1 was also detected in rats fed no *trans* fatty acid, the control group.

DISCUSSION

The studies on the effects of glyceride structure on absorption and metabolism have mainly been focused on structured triacylglycerols composed of medium-chain fatty acids and polyunsaturated fatty acids (24–27), and more detailed studies are still required for understanding this issue (28). The experimental procedure used to study fatty acid absorption may not be physiological, and there is some limitation to the interpretation of the results. However, the observation that the net absorption rate of triacylglycerol was quantitative and that of cholesterol was around 50% supports the usefulness of the current procedure (15,29). Vahouny *et al.* (30) established this procedure after a systemic examination of the experimental conditions.

In the present study, the fatty acid absorption was largely uninfluenced by randomization. However, the apparent absorption rate of *trans* fatty acid seemed to be slightly lower

TABLE 6
Effects of Randomization of Partially Hydrogenated Corn Oil on Fatty Acid Compositons of Liver Triacylglycerol and Phospholipids[a]

	Fatty acid (wt%)									
Lipid fraction	14:0	16:0	16:1	18:0	*t*-18:1	*c*-18:1	18:2n-6	20:4n-6	22:5n-6	22:6n-3
Triacylglycerol										
HOS	1.1 ± 0.3[b]	34.5 ± 1.7[b]	8.9 ± 1.9[b]	1.7 ± 0.3	—	52.5 ± 2.9[b]	2.1 ± 0.5	—	—	—
PHC	1.4 ± 0.2[b]	37.8 ± 2.7[c]	10.4 ± 1.0[c]	1.6 ± 0.3	3.1 ± 0.8	43.9 ± 3.1[c]	1.1 ± 0.7	—	—	—
RPHC	1.5 ± 0.2[c]	38.8 ± 1.7[c]	11.0 ± 1.0[c]	1.7 ± 0.2	3.1 ± 0.2	42.3 ± 2.0[c]	1.0 ± 0.7	—	—	—
Phosphatidylcholine										
HOS	—	18.8 ± 1.0	1.5 ± 0.2[b]	21.0 ± 2.0[b]	—	12.5 ± 0.5[b]	6.3 ± 1.2[b]	30.0 ± 3.7[b]	3.8 ± 0.5[b]	4.6 ± 0.6
PHC	—	17.8 ± 1.3	2.9 ± 0.2[c]	16.3 ± 2.0[c]	5.7 ± 2.3	15.9 ± 0.5[c]	12.4 ± 1.9[c]	20.2 ± 3.8[c]	1.9 ± 0.4[c]	4.2 ± 0.6
RPHC	—	17.3 ± 2.3	3.1 ± 0.3[c]	17.2 ± 0.9[c]	5.1 ± 0.6	15.3 ± 1.2[c]	12.6 ± 1.5[c]	19.3 ± 1.5[c]	2.3 ± 0.3[c]	4.0 ± 0.7
Phosphatidylethanolamine										
HOS	—	14.0 ± 1.3	1.1 ± 0.1	23.6 ± 1.0[b]	—	5.8 ± 0.8[b]	0.8 ± 0.5[b]	33.1 ± 1.8	8.4 ± 0.1[b]	12.0 ± 1.2
PHC	—	14.0 ± 2.0	1.2 ± 0.2	19.9 ± 2.0[c]	5.4 ± 2.0	7.3 ± 1.0[c]	2.1 ± 1.2[c]	33.3 ± 2.0	4.5 ± 1.3[c]	12.3 ± 1.5
RPHC	—	12.8 ± 2.0	1.6 ± 0.3	19.6 ± 1.5[c]	5.1 ± 0.7	6.6 ± 0.4[c]	1.7 ± 0.9[c]	32.8 ± 0.8	5.5 ± 1.0[c]	12.2 ± 1.6

[a]Values are means ± SE of eight rats. See Table 2 for abbreviations.
[b,c]Values with different letters are significantly different at $P < 0.05$.

TABLE 7
Effects of Randomization of Partially Hydrogenated Corn Oil on Fatty Acid Compositions of Fecal Lipids[a]

	Fatty acid (wt%)						
Dietary group	16:0	18:0	*t*-18:1	*c*-18:1	18:2	18:3	20:1
HOS	39.3 ± 1.4[b]	27.6 ± 0.3[b]	2.9 ± 0.6[b]	19.8 ± 2.2	1.3 ± 0.1	1.1 ± 0.1	1.7 ± 0.2
PHC	23.7 ± 2.8[c]	14.3 ± 1.2[c]	37.2 ± 1.2[c]	19.9 ± 1.4	1.6 ± 0.2	1.0 ± 0.3	1.1 ± 0.6
RPHC	20.0 ± 2.0[c]	14.6 ± 1.7[c]	39.7 ± 2.3[c]	21.2 ± 1.0	1.6 ± 0.1	0.6 ± 0.3	1.0 ± 0.5

[a]Values are means ± SE of eight rats. See Table 2 for abbreviations.
[b,c]Values with different letters are significantly different at $P < 0.05$.

than in *cis* fatty acid (93.1 ± 6.8 vs. 94.8 ± 5.2% for PHC and 89.2 ± 8.2 vs. 93.6 ± 4.7% for randomized PHC) as observed for the free fatty acids (31). Because of a less specific positional distribution of fatty acids in triacylglycerol in PHC used in the present study, the effects of randomization on lipid absorption were rather marginal. Thus, the lymphatic fatty acid absorption was largely unmodified after randomization, although it tended to be slightly higher in randomized fat (96.8 ± 9.0 vs. 104 ± 12%). A more distinct improvement of fat absorption has been reported in randomized saturated fat (8). The metabolic significance of this small difference is not known, but the cumulative effect may not be insignificant. Once absorbed, some degree of rearrangement of the positional distribution of fatty acid has been observed when long-chain saturated fatty acids were given as triacylglycerol (3,27) while the randomization did not influence cholesterol absorption.

The fatty acid composition of adipose tissue was similar between two hydrogenated fat groups and reflected the composition of lymph lipids. However, the proportion of palmitic acid in the 2-position of adipose triacylglycerol in rats fed *trans* monoene was significantly higher than in those fed *cis* monoene, despite the same dietary level of palmitic acid. In contrast, the proportion of palmitic acid in whole triacylglycerol was not different among dietary fats. This observation, together with a significant increase in palmitoleic acid at the 2-position, suggests a change in the molecular species of adipose tissue triacylglycerol due to feeding *trans* fatty acid. The implications of this structural change are not clear, but it appears that not all fatty acids are released from and/or incorporated into the adipose tissue at the same rate (32).

The fatty acid composition data of liver phospholipids indicated a considerable interference by *trans* acid with the metabolic pathway leading from linoleic acid to arachidonic acid, in agreement with previous observation (33). However, this change may not influence eicosanoid production as indicated by Zevenbergen and Hademan (34), who showed that in rats 2 energy % linoleic acid is sufficient to prevent effects of dietary *trans* fatty acids on eicosanoid production. The proportion of docosapentaenoic acid (n-6) also decreased after feeding hydrogenated fats. This pentane can accumulate when dietary supply of n-3 polyunsaturated fatty acids is deficit (35). Since the proportion of docosahexaenoic acid (n-3) was not influenced by the type of dietary fats, the reduction of the pentane acid suggests a diverse effect of *cis* and *trans* monoenes on n-3 polyunsaturated fatty acid metabolism.

Although the present study failed to show a clear-cut effect of randomization of *trans* acid-containing fat on fatty acid absorption, it increased serum cholesterol significantly and, to a lesser extent, liver cholesterol as well. Thus, there is a possibility that a marginal increase in intestinal fatty acid absorption cumulatively influences lipid metabolism. In contrast, nonrandomized fat was not hypercholesterolemic as reported previously (33). The increase in liver weight after feeding partially hydrogenated fats may, in part, be attributed to an accumulation of triacylglycerol and phospholipid (Table 4). Thus, the randomization may have an undesirable effect on lipid metabolism. The reduction of heart weight by dietary *trans* fats has not been observed before, and at present it is difficult to explain the observed effect of *trans* fat.

Holman and co-workers (36) indicated that about 4% energy of linoleic acid is required for prevention of disturbance by *trans* fatty acid of polyunsaturated fatty acid composition in tissue phospholipids. Although 2% energy was shown to be enough in other studies (33,34), our rats may have been marginally essential fatty acid-deficient. Hence, the results of tissue weights, cholesterol levels, and phospholipid fatty acids may need to be evaluated in light of this possibility.

In conclusion, our results did not support the idea that randomization of PHC leads to beneficial effect on various lipid parameters, but rather that it enhances the hypercholesterolemic propensityof dietary *trans* fatty acids. There may be cumulative untoward effects when randomized fat was consumed regularly. Since randomization simultaneously causes changes in the distribution of fatty acids other than *trans* fatty acids, particularly palmitic acid, it is difficult to attribute the observed effect entirely to changes in the positional distribution of a single fatty acid.

REFERENCES

1. Elson, C.E., Dugan, L.R., Jr., Bratzler, J., and Pearson, A.M. (1966) Effect of Isoessential Fatty Acid Lipids from Animal and Plant Sources on Cholesterol Levels in Mature Male Rats, *Lipids 1*, 322–324.
2. Yamamoto, I., Sugano, M., and Wada, M. (1971) Hypocholesterolemic Effect of Animal and Plant Fats in Rats, *Atherosclerosis 13*, 171–184.
3. Kritchevsky, D. (1988) Effects of Triglyceride Structure on Lipid Metabolism, *Nutr. Rev. 46*, 177–181.
4. Sugano, M., Ikeda, I., Imasato, Y., Nakayama, M., and Yoshida, K. (1990) Is Triglyceride Structure of Palm Oil Responsible for Its Characteristic Effect on Lipid Metabolism? *Life Sci. Adv. 9*, 21–25.

5. Murakami, T., Chimi, K., Kanematsu, H., Niiya, I., Shimura, M., Mizutani, H., and Hirai, C. (1991) Effect of Processed Oils and Fats on Cholesterol Metabolism II. Effects of Interesterified Palm Oil, *Jpn. J. Oil Chem. Soc. 40*, 114–120.
6. Murakami, T., Shimizu, M., Chimi, K., Mizutani, H., Kanematsu, H., Niiya, I., and Hirai, C. (1992) Effect of Processed Oils and Fats on Cholesterol Metabolism V. Effects of Lard and Its Blend with Palm Olein and Their Randomized Oil, *Jpn. J. Oil Chem. Soc. 41*, 530–537.
7. Schrijver, R.D., Vermeulen, D., and Viaene, E. (1991) Lipid Metabolism Responses in Rats Red Beef Tallow, Native or Randomized Fish Oil and Native or Randomized Peanut Oil, *J. Nutr. 121*, 948–955.
8. Lien, E.L., Yuhas, R.J., Boyle, F.G. and Tomarelli, R.M. (1993) Corandomization of Fats Improbes Absorption in Rats, *J. Nutr. 123*, 1859–1867.
9. Mensink, R.P., and Katan, M.B. (1990) Effect of Dietary *trans* Fatty Acids on High-Density and Low-Density Lipoprotein Cholesterol Levels in Healthy Subjects, *N. Engl. J. Med. 323*, 439–445.
10. Mensink, R.P., Zock, P.L., Katan, M.B., and Hornstra, G. (1992) Effect of Dietary *cis* and *trans* Fatty Acids on Serum Lipoprotein[a] Levels in Humans, *J. Lipid Res. 33*, 1493–1501.
11. Nestel, P.J., Noakes, M., Belling, B., McArthur, R., Clifton, P.M., and Abbey, M. (1992) Plasma Lipoprotein Lipid and Lp[a] Changes with Substitution of Elaidic Acid for Oleic Acid in the Diet, *J. Lipid Res. 33*, 1029–1036.
12. Zock, P.L., and Katan, M.B. (1992) Hydrogenation Alternatives: Effect of *trans* Fatty Acid and Stearic Acid Versus Linoleic Acid on Serum Lipids and Liporotein in Humans, *J. Lipid Res. 33*, 399–410.
13. Nelson, G.J., and Ackman, R.G. (1988) Absorption and Transport of Fat in Mammals with Emphasis on n-3 Polyunsaturated Fatty Acids, *Lipids 23*, 1005–1014.
14. Paquet, C. (1979) Standard Methods for the Analysis of Oils, Fats and Derivatives, 6th edn., Part 1, pp. 84–88, Pergamon Press, New York.
15. Ikeda, I., Imasato, Y., Nagao, H., Sasaki, E., Sugano, M., Imaizumi, K., and Yazawa, K. (1993) Lymphatic Transport of Eicosapentaenoic and Docosahexaenoic Acids as Triglyceride, Ethyl Ester and Free Acid, and Their Effect on Cholesterol Transport in Rats, *Life Sci. 52*, 1371–1379.
16. American Institute of Nutrition (1977) Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies, *J. Nutr. 107*, 1340–1348.
17. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
18. Ikeda, I., Tomari, Y., and Sugano, M. (1989) Interrelated Effects of Dietary Fiber and Fat on Lymphatic Cholesterol and Triglyceride Absorption in Rats, *J. Nutr. 119*, 1383–1387.
19. Sugano, M., Ryu, K., and Ide, T. (1984) Cholesterol Dynamics in Rats Fed *cis*- and *trans*-Octadecenoate in the Form of Triglyceride, *J. Lipid Res. 25*, 474–485.
20. Takamura, H., Narita, H., Park, H.J., Tanaka, K., Matuura, T., and Kito, M. (1987) Differential Hydrolysis of Phospholipid Molecular Species During Activation of Human Platelets with Thrombin and Collagen, *J. Biol. Chem. 262*, 2262–2269.
21. Rouser, G., Siakotos, A.N., and Fleischer, S. (1966) Quantitative Analysis of Phospholipids by Thin-Layer Chromatography and Phosphorus Analysis of Spots, *Lipids 1*, 85–86.
22. Kamer, J.H., Bokhel Huinink, H., and Weyrs, H.A. (1949) Rapid Method for the Determination of Fat in Feces, *J. Biol. Chem. 177*, 347–355.
23. Duncan, D.B. (1955) Multiple Range and Multiple F Tests, *Biometrics 1*, 1–42.
24. Ikeda, I., Tomari, Y., Sugano, M., Watanabe, S., and Nagata, J. (1991) Lymphatic Absorption of Structured Glycerolipids Containing Medium-Chain Fatty Acids and Linoleic Acid and Their Effect on Cholesterol Absorption, *Lipids 26*, 369–373.
25. Gollaher, C.J., Swenson, S., Mascioli, E.A., Babayan, V.K., Blackburn, G.L., and Bistrian, B.R. (1992) Dietary Fat Level as Determinant of Protein-Spearing Actions of Structured Triglycerides, *Nutrition 8*, 348–353.
26. Jensen, M., Christensen, M.S., and Hoy, C.-E. (1994) Intestinal Absorption of Octanoic, Decanoic and Linoleic Acids: Effect of Triglyceride Structure, *Ann. Nutr. Metab. 39*, 104–116.
27. Jensen, G.L., McGarvey, N., Taraszewski, R., Wixson, S.K., Seidner, D.L., Pai, T., Yeh, Y.-Y., Lee, T.W., and DeMichele, S.J. (1994) Lymphatic Absorption of Enterally Fed Structured Triacylglycerol vs. Physical Mix in a Canine Model, *Am. J. Clin. Nutr. 60*, 518–524.
28. Small, D.M. (1991) The Effects of Glyceride Structure on Absorption and Metabolism, *Ann. Rev. Nutr. 11*, 413–434.
29. Ikeda, I., Tanaka, K., Sugano, M., Vahouny, G., and Gallo, L.L. (1988) Inhibition of Cholesterol Absorption in Rats by Plant Sterols, *J. Lipid Res. 29*, 1573–1582.
30. Vahouny, G., Fawal, I., and Treadwell, C.R. (1957) Factors Facilitating Cholesterol Absorption from the Intestine *via* Lymphatic Pathways, *Am J. Physiol. 188*, 342–346.
31. Bernard, A., Echinard, B., and Carlier, H. (1987) Differential Intestinal Absorption of Two Fatty Acid Isomers: Elaidic and Oleic Acids, *Am. J. Physiol. 253*, G751–G759.
32. Cunnane, C.S., Chen, Z.-Y., Yang, J., Liede, A.C., Hamadeh, M., and Crawford, M.A. (1991) α-Linolenic Acid in Humans: Direct Functional Role or Dietary Precursor? *Nutrition 7*, 437–439.
33. Sugano, M., Watanabe, M., Yoshida, K., Miyamoto, M., and Kritchevsky, D. (1989) Influence of Dietary *cis* and *trans* Fat on DMH-induced Colon Tumors, Steroid Excretion and Eicosanoid Production in Rats Prone to Colon Cancer, *Nutr. Cancer 12*, 177–187.
34. Zevenbergen, J.L., and Hademan, E. (1989) Lack of Effects of *trans* Fatty Acids on Eicosanoid Biosynthesis with Adequate Intakes of Linoleic Acid, *Lipids 24*, 555–563.
35. Ruiz-Gutierrez, V., Molina, M.T., and Vazquez, C.M. (1990) Comparative Effects of Feeding Different Fats on Fatty Acid Composition of Major Individual Phospholipids of Rat Hearts, *Ann. Nutr. Metab. 34*, 350–358.
36. Holman, R.T., Mahfouz, M.M., Lawson, L.D., and Hill, E.G. (1981) in *Dietary Fats and Health* (Perkins, E.G., and Visek, W.J., eds.) pp. 320–340, American Oil Chemists' Society, Champaign.

[Received March 7, 1995, and in final revised form August 16, 1995; Revision accepted August 17, 1995]

Erythrocyte Fatty Acids of Term Infants Fed Either Breast Milk, Standard Formula, or Formula Supplemented with Long-Chain Polyunsaturates

Maria Makrides, Mark A. Neumann, Karen Simmer and Robert A. Gibson*

Department of Paediatrics & Child Health, Flinders Medical Centre, Bedford Park (Adelaide), South Australia 5042, Australia

ABSTRACT: The purpose of our study was to assess whether a supplement of fish oil (FO) and evening primrose oil (EPO) for formula-fed infants was capable of avoiding reductions in erythrocyte docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) associated with standard formula feeding. Healthy, term infants, whose mothers chose to formula feed, were randomized to either a placebo or supplemented formula for their first 30 wk of life. A reference group of beast-fed infants also was enrolled. Erythrocyte fatty acids were measured by capillary gas chromatography on day 5 and in weeks 6, 16, and 30. Supplementation of formula with 0.36% of total fatty acids as DHA resulted in erythrocyte DHA being maintained at or above breast-fed levels for the entire 30-wk study period, and breast feeding (0.21% DHA) resulted in a modest fall in erythrocyte DHA relative to baseline (day 5) values. The level of erythrocyte DHA in placebo formula-fed infants was halved by week 16. AA levels decreased in all infants in the first six weeks, but the levels in breast- and placebo formula-fed infants increased with age and returned to approximate baseline (day 5) values by 16 and 30 wk of age, respectively. Erythrocyte AA in FO + EPO-supplemented infants remained low and below breast- and placebo formula-fed levels. Our data suggest that dietary supplementation with DHA at 0.36% total fatty acids results in erythrocyte DHA levels above those found in breast-fed infants. EPO supplementation was not effective at maintaining erythrocyte AA when given with FO.

Lipids 30, 941–948 (1995).

Recent studies have postulated that the n-3 long-chain polyunsaturated fatty acid (LCPUFA), docosahexaenoic acid (DHA, 22:6n-3), may be important in the development of optimal neural function in preterm infants. Preterm infants fed diets containing n-3 LCPUFA [breast milk or formulas supplemented with fish oils (FO)] are reported to have improved neural maturation as assessed by electroretinograms, visual evoked potential acuity, and behavioral acuity measures compared with infants fed standard, unsupplemented formula (1–3). However, despite such observations, scientists and clinicians have been hesitant to recommend the addition of FO to infant formulas. Most FO, specifically those that have become routine in clinical trials, contain more eicosapentaenoic acid (EPA, 20:5n-3; ≈15% of total fatty acids) than DHA (≈11% total fatty acids), and have negligible n-6 fatty acids. In contrast, breast milk has more DHA than EPA and also contains n-6 LCPUFA such as arachidonic acid (AA, 20:4n-6). Preterm infants fed FO-supplemented formula have increased erythrocyte levels of EPA and reduced AA relative to infants fed standard formula (4,5) and breast milk (5). Such decreases in AA status of FO-supplemented formula-fed preterm infants have been associated with poorer growth compared with standard formula-fed infants (6).

EPA is the precursor to the three-series eicosanoids, which may suppress some effects of the two-series eicosanoids originating from AA. Although this may be beneficial in certain pathological instances, such as chronic inflammatory or thrombotic conditions, concerns persist about limiting the activity of AA-derived eicosanoids in infancy. For example, in preterm studies, bleeding times of FO-supplemented formula-fed infants are reported to be longer than those for infants fed human milk or unsupplemented formulas at 35–37 wk postconceptional age (5). Although all bleeding times were reported to be within accepted clinical norms, concerns still persist, especially considering the predisposition of preterm infants to intracranial bleeding.

However, most infants are born at term, and most recent research attention has focused on the dietary requirements of the healthy infant. We (7) and others (8,9) have reported that breast-fed term infants have better neural responses than infants fed formula, and postulated that DHA may be associated with these observations, as higher levels of DHA in the erythrocytes of breast-fed infants are associated with improved visual measures (7). The aim of this study was to assess n-3 LCPUFA supplementation of formula for term infants on erythrocyte fatty acid status and visual evoked potential acuity. Data on visual acuity are reported elsewhere (10), and detailed fatty acid results are reported here. We assessed whether FO together with evening primrose oil (EPO) was capable of increasing erythrocyte DHA of formula-fed infants to approximate breast-fed infant levels, without a con-

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; ALA, α-linoleic acid; ANOVA, analysis of variance; DGLA, dihomo-γ-linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPO, evening primrose oil; FO, fish oil; GLA, γ-linoleic acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acids.

Copyright © 1995 by AOCS Press

comitant reduction in erythrocyte AA, over the first 30 wk of infancy. At the time our study was commended, AA was not available for use as a supplement for infant formula, and therefore, we decided to use EPO, which contains γ-linolenic acid (GLA, 18:3n-6), a metabolic precursor of AA, that by-passes the supposed rate-limiting δ6 desaturase enzyme; it was hypothesized that GLA would prevent a fall in erythrocyte AA.

METHODS

Subjects. Women giving birth to healthy, term (37–42 wk gestation) infants at Flinders Medical Centre (Bedford Park, Australia) were eligible for entry into the study. Only women who had no known history of lipid metabolism disorders and insulin-dependent diabetes, and had babies with appropriate weight for gestation were approached to enter the study. Written informed consent was obtained following guidelines stipulated by the Committee on Clinical Investigations (Ethics). The mother's decision to breast or formula feed was not influenced, as enrollment to the study was after she had made her feeding choice.

Dietary treatment. Mothers who intended to formula feed their infants were randomly assigned either a placebo or supplemented formula. Mothers and assessment staff were unaware of the formula type (10). The supplement used was a 1:1 microencapsulated blend of FO + EPO in vacuum sealed sachets, supplied by Scotia Pharmaecuticals (Guildford, United Kingdom). The base formula was donated by Nestle Australia (Sydney, Australia) and was similar in nutrient composition to commercial "Nan," an infant formula (Nestle Australia), except that the fat blend was purely of vegetable origin (10). Mothers in the supplemented group were asked to add the contents of one sachet to 200 mL of formula, and mothers in the placebo group were instructed to add an extra amount of placebo powder to 210 mL of formula (10). The fatty acid compositions of the two study formulas were analyzed before the study and intermittently throughout the study period. The composition was stable and is listed in Table 1. The limited supply of FO + EPO supplement resulted in a smaller number of infants being enrolled in this group.

Mothers who chose to breast feed were encouraged to do so for as long as possible. The predetermined definition of full breast feeding was no formula in the first 16 wk of life and a maximum of 120 mL formula/day from 16 to 30 wk of age. Mothers were telephoned on a weekly basis to inquire about feeding practice, and were offered support. Infants who did not meet the criteria for full breast feeding were excluded. Of 47 mothers recruited with an intent to breast feed, 24 were excluded.

Infants with feeding or settling problems were referred, when appropriate, to a dietitian, pediatrician, or lactation consultant. All mothers received nutrition advice according to National Health & Medical Research Council guidelines with the recommendation that solid foods be introduced between four and six months of age (11). The fatty acids contribution of solid foods was not quantified as common weaning foods contain negligible amounts of LCPUFA (12).

TABLE 1
Fatty Acid Composition of Infant Formulas and Breast Milk of Each Mother During Week 16 of Lactation (data expressed as mean % total fatty acids ± SD)

Fatty acid	Placebo formula (n = 14)[a]	Supplemented formula (n = 12)[a]	Breast milk (n = 23)[b]
Total saturated	50.60 ± 0.32	48.99 ± 0.22	45.33 ± 3.33
Total monosaturated	30.97 ± 0.27	30.75 ± 0.17	38.11 ± 2.66
18:2n-6	16.79 ± 0.08	17.44 ± 0.16	13.92 ± 3.02
18:3n-6	0.05 ± 0.02	0.27 ± 0.03	0.16 ± 0.04
20:3n-6	ND[c]	ND	0.35 ± 0.08
20:4n-6	ND	0.01 ± 0.01	0.40 ± 0.07
22:4n-6	ND	ND	0.07 ± 0.02
22:5n-6	ND	ND	0.03 ± 0.01
Total n-6	16.85 ± 0.09	17.73 ± 0.16	15.13 ± 3.12
18:3n-3	1.58 ± 0.01	1.52 ± 0.02	0.94 ± 0.25
20:5n-3	ND	0.58 ± 0.04	0.07 ± 0.04
22:5n-3	ND	0.07 ± 0.01	0.16 ± 0.04
22:6n-3	ND	0.36 ± 0.03	0.21 ± 0.13
Total n-3	1.58 ± 0.01	2.53 ± 0.07	1.43 ± 0.35

[a]The analysis of infant formula was checked before the commencement of the study and intermittently throughout the study period.
[b]Each analysis represents a pooled breast milk sample collected over seven consecutive days in week 16 of lactation from mothers of fully breast-fed infants.
[c]ND, not detected.

Erythrocyte fatty acid assessment. Blood samples were taken by heel-prick (200 μL) on day 5 (timed to coincide with the routine Guthrie test), 6 wk, 16, and 30 wk, into tubes containing lithium heparin. In the case where consent to participate in the study was attained after the Guthrie test and the mother did not wish a further blood test, no day 5 blood sample was available for analysis. Erythrocytes were separated from plasma, and lipids were extracted, methylated, and quantified following previously reported procedures (13).

Statistical analysis. All data are expressed as mean ± SD unless otherwise stated. Effects of diet and age (time) on erythrocyte fatty acids of fully breast-fed, placebo-, and supplemented formula-fed infants were determined by repeated measures analysis of variance (ANOVA). Significant differences due to either dietary grouping or age were subsequently identified by least significant difference. However, differences in erythrocyte linoleic acid (LA, 18:2n-6), AA, 22:5n-3, and DHA were already established between infants who were breast-fed and those who received formula by day 5 of life (see Table 2). It was decided not to correct/normalize these differences as they were probably dietary-induced and, hence, important. Further, the correction of baseline (day 5) variation, either by expressing the fatty acid data as change from day 5 or including the day 5 values as a constant covariate in the repeated measures ANOVA, would have resulted in the loss of a degree of freedom in the time series (14). Hence, it would not be possible to statistically test for differences between day 5 and 6 wk within each dietary group. This was pertinent, as large shifts in fatty acids, such as AA, LA, DHA, and EPA, occurred in the first six weeks of life and were often related to dietary grouping.

TABLE 2
Day 5 Infant Erythrocyte Fatty Acids (data expressed as mean % total fatty acids ± SD)[a]

Fatty acid	Intend to breast feed (n = 34)	Intend to formula feed (n = 24)
Total saturated	44.82 ± 2.23	44.41 ± 3.20
Total monosaturated	18.88 ± 5.24	19.84 ± 6.80
18:2n-6	4.79 ± 0.88[b]	6.35 ± 0.98[c]
18:3n-6	0.05 ± 006	0.03 ± 0.05
20:3n-6	2.23 ± 0.47	2.46 ± 0.63
20:4n-6	16.19 ± 1.89[b]	14.96 ± 2.22[c]
22:4n-6	4.00 ± 0.62	3.84 ± 0.60
22:5n-6	1.53 ± 0.44	1.49 ± 0.27
Total n-6	28.90 ± 2.70	29.24 ± 3.08
18:3n-3	0.12 ± 0.11	0.13 ± 0.10
20:5n-3	0.13 ± 0.06	0.13 ± 0.12
22:5n-3	0.56 ± 0.12[b]	0.50 ± 0.09[c]
22:6n-3	6.11 ± 0.99[b]	5.31 ± 0.87[c]
Total n-3	6.91 ± 1.09[b]	6.03 ± 0.93[c]

[a]Values without common superscripts indicate significant differences at $P < 0.05$.

Thirteen infants had missing day 5 blood samples because they were enrolled after the routine Guthrie blood test, which would have excluded them from the analysis as they had incomplete sets of serial data. In all these cases, consent to participate in the study was obtained on day 5 or 6 after the routine Guthrie test, and mothers did not wish that extra blood be taken from their babies. As this was a random occurrence at the beginning of the study before any dietary treatment had been administered (five breast-fed infants, eight formula-fed infants), it was rationalized that these missing values could be replaced by the appropriate day 5 erythrocyte fatty acid means (listed in Table 2). Frequency distributions for each erythrocyte fatty acid were checked before and after replacement of missing values, and little change was noted in the variance. Further, the repeated measured ANOVA on each erythrocyte fatty acid was performed both with and without the missing values replaced, and again, little difference was noted. All analyses were completed using SPSS for Windows 6.0 (SPSS Inc., Chicago, IL).

RESULTS

The subjects are described in Table 3. Infants from all groups were of similar gestation, weight, length, and head circumference at birth. A total of 13 subjects were enrolled into the supplemented-formula group as only a limited supply of EPO + FO supplement was available. The data presented here are from infants with complete sets of serial fatty acid measures (21/23 fully breast-fed infants; 18/19 placebo formula-fed infants; 12/13 supplemented formula-fed infants; Table 4). All four missing values were a result of incomplete blood analysis at 30 wk.

Erythrocyte n-3 fatty acids. There was a modest reduction in DHA in breast-fed infants that achieved statistical significance at 30 wk of age (Fig. 1 and Table 4). In contrast, DHA levels in placebo formula-fed infants rapidly declined for 16 wk (53% decrease), slowing thereafter so that an apparent further decline (9%) at 30 wk did not reach statistical significance. Supplementation of infant formula with FO (0.36% total fatty acids as DHA) resulted in DHA levels being elevated above those of breast-fed infants at 16 and 30 wk (Fig. 1 and Table 4).

TABLE 3
Description of Subjects

Parameter	Fully breast-fed	Placebo formula-fed	Supplemented formula-fed
n	23	19	13
Gestation (wk ± SD)	39.8 ± 1.1	39.6 ± 1.2	39.1 ± 1.7
Male/female ratio	9:14	8:11	9:4
Apgar at 5 min (score ± SD)	9.5 ± 0.5	9.3 ± 0.6	9.1 ± 0.8
Birth weight (g ± SD)	3589 ± 360	3650 ± 416	3288 ± 525
Birth length (cm ± SD)	51.0 ± 1.9	51.2 ± 2.1	50.2 ± 2.8
Birth head circumference (cm ± SD)	35.1 ± 1.0	35.2 ± 1.2	34.4 ± 2.2

Although erythrocyte EPA in breast- and placebo formula-fed infants significantly increased between day 5 and 16 wk, EPA levels remained below 0.4% of total fatty acids for the whole of the study's period (Fig. 2 and Table 4). Such results were in direct contrast to those observed for infants fed the FO + EPO supplement, where there was a tenfold rise in EPA levels between day 5 and week 30 (Fig. 2 and Table 4), with a maximum EPA level of ≈2% of total fatty acids. Erythrocyte 22:5n-3 increased in all infants with age, but was greatest in FO + EPO-supplemented formula-fed infants (Table 4). α-Linolenic acid (ALA, 18:3n-3) accounted for ≈0.20% of total fatty acids in all dietary groups at all assessment ages.

Erythrocyte n-6 fatty acids. A decrease in AA between day 5 and week 6 was observed in all infants (12% in fully breast-fed infants, 20% in placebo formula-fed infants, and 28% in supplemented formula-fed infants; Fig. 3 and Table 4). Thereafter, both the breast and placebo formula groups demonstrated significant shifts toward baseline levels, so that breast-fed infants attained day 5 levels by 16 wk and there was no difference in the level of AA between breasts and placebo formula-fed infants at 30 wk. Erythrocyte AA in the supplemented formula group remained below that of infants fed either breast milk or placebo formula at all time points except day 5.

Erythrocyte LA increased in all dietary groups from a day 5 level of 5–6% to 8–12% of total fatty acids at week 6 (Fig. 4 and Table 4). LA was greatest in placebo formula-fed infants, rather than in the breast and supplemented formula groups at 6, 16, and 30 wk. Between 16 and 30 wk of age, there was a modest, but significant, increase in the level of erythrocyte LA in all infants, and that was independent of their dietary grouping.

All infants demonstrated significant reductions in 22:4n-6 and 22:5n-6 with age, but were most pronounced in the FO +

TABLE 4
Infant Erythrocyte Fatty Acids (data expressed as mean % total fatty acids ± SD)[d]

	Day 5			Week 6			Week 16		
Fatty acid	Breast (n = 21)	Placebo (n = 18)	Supplemented (n = 12)	Breast (n =21)	Placebo (n = 18)	Supplemented (n =12)	Breast (n = 21)	Placebo (n = 18)	Supplemented (n = 12)
Total saturated	44.7 ± 2.0	44.4 ± 2.5	44.0 ± 3.1	44.1 ± 2.4	44.4 ± 2.7	43.3 ± 3.7	43.6 ± 1.9	42.3 ± 2.5	43.5 ± 2.7
Total monosaturated	19.3 ± 5.2	20.0 ± 5.5	20.2 ± 6.9	18.9 ± 4.7	19.4 ± 4.3	21.1 ± 8.1	18.4 ± 4.4	21.5 ± 4.8	20.3 ± 5.2
18:2n-6	$4.9 \pm 1.0^{a,1}$	$6.4 \pm 0.6^{b,1}$	$6.4 \pm 1.1^{b,1}$	$8.5 \pm 0.8^{a,2}$	$11.9 \pm 1.0^{b,2}$	$10.7 \pm 1.1^{c,2}$	$8.7 \pm 0.9^{a,2}$	$11.7 \pm 1.0^{b,2}$	$10.5 \pm 0.6^{c,2}$
18:3n-6	ND	ND	ND	0.1 ± 0.2	0.1 ± 0.1	ND	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:3n-6	$2.3 \pm 0.4^{a,1}$	$2.5 \pm 0.6^{a,1}$	$2.4 \pm 0.5^{a,1}$	$2.1 \pm 0.4^{a,2}$	$2.1 \pm 0.5^{a,2}$	$1.9 \pm 0.4^{a,2}$	$1.7 \pm 0.3^{a,3}$	$2.1 \pm 0.4^{b,2}$	$1.9 \pm 0.3^{a,b,2}$
20:4n-6	$16.0 \pm 2.1^{a,1}$	$14.8 \pm 1.7^{b,1}$	$15.1 \pm 2.4^{a,b,1}$	$14.1 \pm 1.4^{a,2}$	$11.8 \pm 1.2^{b,2}$	$11.0 \pm 1.8^{b,2}$	$15.1 \pm 1.4^{a,1}$	$12.9 \pm 1.2^{b,3}$	$10.2 \pm 1.0^{c,2}$
22:4n-6	$3.9 \pm 0.6^{a,1}$	$3.8 \pm 0.4^{a,1}$	$3.7 \pm 0.7^{a,1}$	$3.7 \pm 0.6^{a,1,2}$	$3.5 \pm 0.5^{a,2}$	$2.8 \pm 0.5^{b,2}$	$3.5 \pm 0.5^{a,2}$	$3.6 \pm 0.4^{a,2}$	$1.7 \pm 0.3^{b,3}$
22:5n-6	$1.5 \pm 0.5^{a,1}$	$1.5 \pm 0.2^{a,1}$	$1.4 \pm 0.3^{a,1}$	$1.3 \pm 0.3^{a,2}$	$1.2 \pm 0.3^{a,2}$	$0.9 \pm 0.3^{b,2}$	$0.8 \pm 0.3^{a,3}$	$0.9 \pm 0.1^{a,3}$	$0.3 \pm 0.1^{b,3}$
Total n-6	28.8 ± 3.0^{a}	29.2 ± 2.4^{a}	29.2 ± 3.3^{a}	30.0 ± 2.3^{a}	30.6 ± 2.1^{a}	27.5 ± 3.6^{b}	30.2 ± 2.5^{a}	31.5 ± 2.2^{a}	24.9 ± 1.7^{b}
18:3n-3	0.1 ± 0.2^{1}	0.1 ± 0.1^{1}	0.1 ± 0.1^{1}	0.1 ± 0.1^{1}	0.2 ± 0.1^{2}	0.2 ± 0.1^{2}	$0.2 \pm 0.1^{1,2}$	0.2 ± 0.1^{2}	0.2 ± 0.1^{2}
20:5n-3	$0.1 \pm 0.1^{a,b,1}$	$0.1 \pm 0.1^{a,1}$	$0.2 \pm 0.1^{b,1}$	$0.2 \pm 0.1^{a,1}$	$0.1 \pm 0.1^{a,1,2}$	$1.2 \pm 0.2^{b,2}$	$0.3 \pm 0.1^{a,2}$	$0.2 \pm 0.1^{b,2}$	$1.9 \pm 0.2^{c,3}$
22:5n-3	$0.5 \pm 0.1^{a,1}$	$0.5 \pm 0.1^{a,1}$	$0.5 \pm 0.1^{a,1}$	$1.0 \pm 0.2^{a,2}$	$0.7 \pm 0.1^{b,2}$	$1.2 \pm 0.3^{c,2}$	$1.9 \pm 0.2^{a,3}$	$1.7 \pm 0.5^{b,3}$	$2.8 \pm 0.4^{c,3}$
22:5n-3	$5.9 \pm 0.8^{a,1}$	$5.3 \pm 0.6^{b,1}$	$5.3 \pm 1.0^{b,1}$	$5.5 \pm 0.8^{a,1}$	$4.2 \pm 0.7^{b,2}$	$5.4 \pm 1.1^{a,1}$	$5.4 \pm 1.0^{a,1}$	$2.5 \pm 0.3^{b,3}$	$6.3 \pm 0.7^{c,2}$
Total n-3	$6.7 \pm 0.9^{a,1}$	$5.9 \pm 0.6^{b,1}$	$6.1 \pm 1.0^{a,b,1}$	$6.8 \pm 0.9^{a,1}$	$5.3 \pm 0.7^{b,1}$	$7.9 \pm 1.4^{c,2}$	$7.8 \pm 1.1^{a,2}$	$4.6 \pm 0.8^{b,2}$	$11.2 \pm 1.1^{c,3}$

	Week 30			Statistics (*P*-value)		
Fatty acid	Breast (n = 21)	Placebo (n = 18)	Supplemented (n = 12)	Diet	Age	Diet × age
Total saturated	43.4 ± 4.0	43.5 ± 1.2	44.0 ± 1.5	NS	NS	NS
Total monosaturated	20.0 ± 3.7	18.4 ± 2.1	18.7 ± 3.1	NS	NS	NS
18:2n-6	$9.3 \pm 1.4^{a,3}$	$13.1 \pm 1.3^{b,3}$	$11.3 \pm 0.6^{c,3}$	<0.001	<0.001	<0.001
18:3n-6	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	—	—	—
20:3n-6	$1.4 \pm 0.3^{a,4}$	$2.1 \pm 0.4^{b,2}$	$1.9 \pm 0.3^{b,2}$	<0.05	<0.001	<0.001
20:4n-6	$14.6 \pm 2.5^{a,1,2}$	$13.7 \pm 1.1^{a,3}$	$10.5 \pm 1.0^{b,2}$	<0.001	<0.001	<0.001
22:4n-6	$3.1 \pm 0.6^{a,3}$	$3.6 \pm 0.4^{b,2}$	$1.5 \pm 0.3^{c,3}$	<0.001	<0.001	<0.001
22:5n-6	$0.7 \pm 0.2^{a,4}$	$0.9 \pm 0.1^{b,3}$	$0.2 \pm 0.0^{c,3}$	<0.001	<0.001	<0.001
Total n-6	29.4 ± 4.7^{a}	33.7 ± 1.6^{b}	25.6 ± 1.3^{c}	<0.001	NS	<0.001
18:3n-3	0.2 ± 0.1^{2}	0.2 ± 0.1^{2}	0.2 ± 0.0^{2}	NS	<0.005	NS
20:5n-3	$0.3 \pm 0.2^{a,2}$	$0.2 \pm 0.1^{b,2}$	$2.1 \pm 0.2^{c,4}$	<0.001	<0.001	<0.001
22:5n-3	$1.9 \pm 0.4^{a,3}$	$1.9 \pm 0.3^{a,4}$	$3.1 \pm 0.4^{b,4}$	<0.001	<0.001	<0.001
22:6n-3	$4.7 \pm 1.4^{a,2}$	$2.0 \pm 0.4^{b,3}$	$6.3 \pm 0.7^{c,2}$	<0.001	<0.001	<0.001
Total n-3	$7.1 \pm 1.7^{a,1}$	$4.3 \pm 0.6^{b,2}$	$11.7 \pm 1.1^{c,3}$	<0.001	<0.001	<0.001

[d]Superscripts a,b, and c indicate significant differences between dietary groups at each assessment age. Superscripts 1, 2, 3 and 4 indicate significant differences between assessment ages for each dietary group.ND, not detected <0.05% total fatty acid. NS, not significant-no repeated measures analysis of variance.

EPO formula group (Table 4). This contributed to the fact that the level of both 22-C n-6 (22:4 and 22:5) fatty acids were reduced in supplemented formula-fed infants relative to the breast milk and placebo formula-fed groups at 6, 16, and 30 wk (Table 4).

The level of GLA in erythrocytes was <0.08% of total fatty acids in all dietary groups. In fact, no complete cases were available for the repeated measures ANOVA, highlighting the negligible accumulation of GLA in erythrocytes. Conversely, dihomo-γ-linoleic acid (DGLA) accounted for ≈2% of total erythrocyte fatty acids. DGLA modestly decreased between day 5 and 6 wk with all dietary groups having similar levels at 6 wk. Breast-fed infants demonstrated further declines with age and had lower levels of DGLA at 30 wk compared to both supplemented and placebo formula groups (Table 4).

DISCUSSION

The primary aim of our study was to assess the efficacy of prolonged fish oil (DHA) supplementation on the erythrocyte fatty acid profiles of formula-fed term infants. Infant formula with 0.36% DHA resulted in erythrocyte DHA above breast-fed levels at 16 and 30 wk of age. From this point of view, it is interesting to note that the average level of DHA in the milk of Australian mothers has apparently declined from 0.32 to 0.21% total fatty acids over the last decade (15). Furthermore, the current breast milk level of 0.21% resulted in a decrease in erythrocyte DHA over the 30-wk study period, suggesting that a dietary level of 0.21% DHA may not be enough to maintain baseline (day 5) levels. This raises an important issue regarding the definition of optimal or ideal DHA requirements and whether these are best represented by DHA content of breast milk or the level required to match and maintain DHA at birth. For example, as breast milk DHA is largely dependent on the DHA content of the maternal diet (16), it could be argued that such a variable benchmark is inappropriate for use as a dietary requirement. Similarly, infant DHA status at birth also may be affected by maternal diet, although there have been few investigations on the effect of diet in pregnancy on infant erythrocyte fatty acids. Presuming that

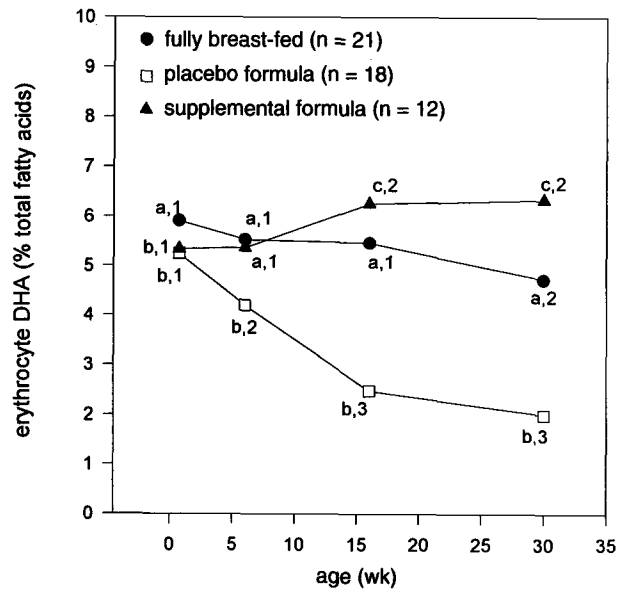


FIG. 1. Erythrocyte docosahexaenoic acid (DHA) as a function of age. *Different alphabetic symbols indicate differences between diet groups* at each age. Different numeric symbols indicate difference between ages for each diet group. Symbols represent mean values for dietary groups.

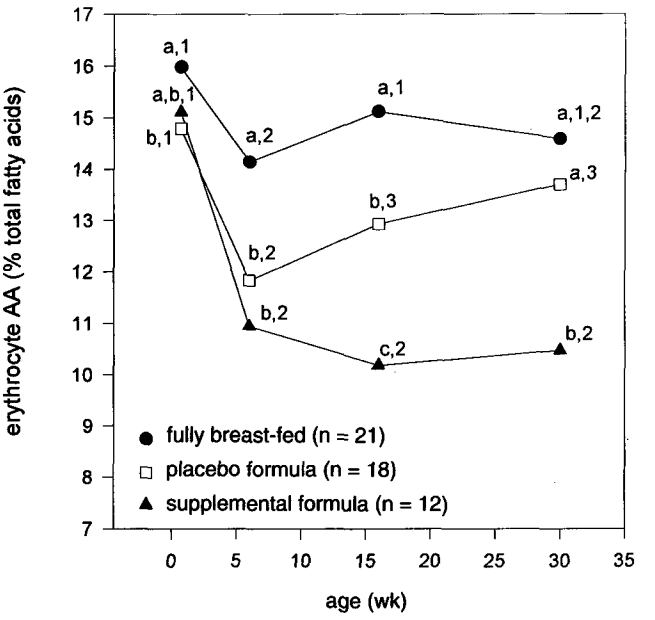


FIG. 3. Erythrocyte arachidonic acid (AA) as a function of age. Different alphabetic symbols indicate differences between diet groups at each age. Different numeric symbols indicate difference between ages for each diet group. Symbols represent mean values for dietary groups.

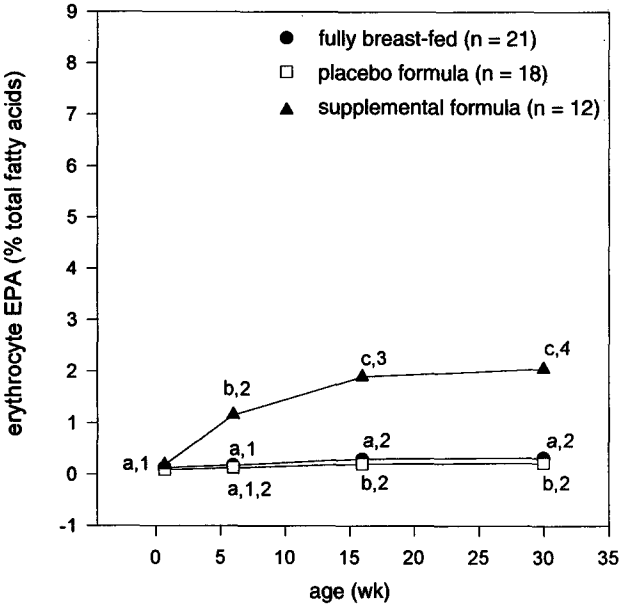


FIG. 2. Erythrocyte eicosapentaenoic acid (EPA) as a function of age. Different alphabetic symbols indicate differences between diet groups at each age. Different numeric symbols indicate difference between ages for each diet group. Symbols represent mean values for dietary groups.

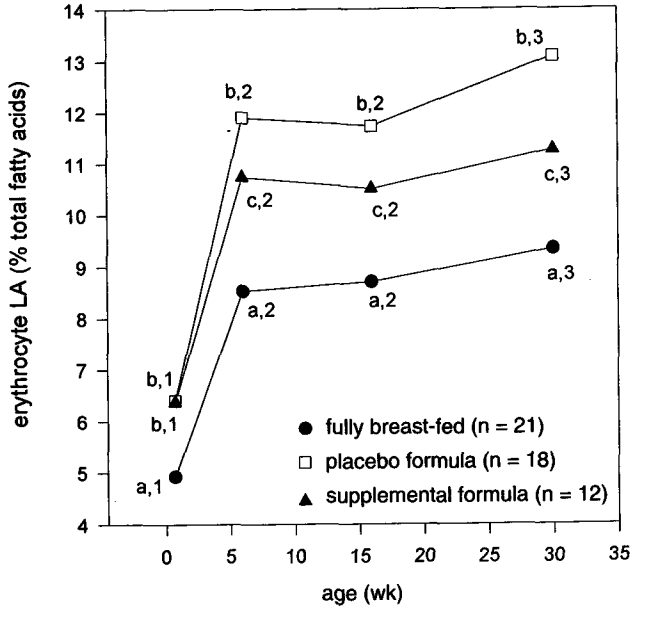


FIG. 4. Erythrocyte linoleic acid (LA) as a function of age. Different alphabetic symbols indicate differences between diet groups at each age. Different numeric symbols indicate difference between ages for each diet group. Symbols represent mean values for dietary groups.

day 5 erythrocyte DHA levels of infants initially breast-fed reflect an ideal intake of DHA, our data would suggest that dietary supplementation of the order of 0.30% DHA would be necessary to maintain this level throughout the first 30 wk of life.

The decline in erythrocyte DHA of placebo formula-fed infants was one of the most notable shifts in fatty acid status observed. Given the postulated importance of DHA, it is natural to question why this reduction was so profound, even though DHA was not supplied in the diets of placebo for-

mula-fed infants. Other LCPUFA, such as AA, that also were not supplied in the formula demonstrated a reduction that was eightfold smaller than for DHA. These findings may, in part, be explained by the regulation of LCPUFA biosynthesis. For example, in the n-3 metabolic pathway, it is proposed that there are two substrates for the δ6 desaturase, namely 18:3n-3 (→ 18:4n-3) and 24:5n-3 (→ 24:6n-3) (17,18). As the δ6 desaturase is often referred to as the rate-limiting enzyme in PUFA biosynthesis, it is interesting to speculate that DHA synthesis is limited by competitive inhibition of a δ6 desaturase by at least four substrates, 18:3n-3, 24:5n-3, 18:2n-6, and 18:1n-9. This therefore implies that synthesis of DHA from ALA is impeded not only by two rate-limiting steps, but also by the presence of low levels of substrate (ALA) and large amounts of LA (18:2n-6) in infant formulas. Indeed, a recent study investigating the effect of dietary LA on the conversion of ALA and LA to their respective LCPUFA demonstrated that the conversion of ALA to DHA was approximately halved by high dietary LA (19). A feeding study in which the dietary LA/ALA ratio of adults was lowered from 18:1 to 0.6:1 increased levels of EPA in plasma and neutrophils, but failed to show significant differences in DHA status (20). However, whatever the rate of DHA synthesis in standard formula-fed infants, it is clearly inadequate to match the erythrocyte levels of DHA seen in breast-fed infants.

Supplementation of formula with FO + EPO also resulted in notable increases in erythrocyte EPA and 22:5n-3 in these infants compared to values seen in infants fed either breast milk or placebo formula. Furthermore, the proportion of erythrocyte 22:5n-3 remained greater than the proportion of erythrocyte EPA, although the supplemented formula contributed more dietary EPA (0.58% total fatty acids) than 22:5n-3 (0.07% total fatty acids). Similar effects also were observed in the erythrocyte lipids of FO-supplemented formula-fed preterm infants (5), suggesting either a preference for the accumulation of 22:5n-3 over EPA in erythrocyte membranes or a slow conversion of 22:5n-3 to DHA. This would further add support for the presence of a limiting δ6 desaturase at this point in the metabolic pathway (17).

Following a rapid decline in erythrocyte AA levels during the first six weeks, placebo formula-fed infants demonstrated a significant trend toward the accumulation of AA between 6 and 30 wk, despite having no direct dietary source. Erythrocyte levels of AA in preterm infants fed unsupplemented formula containing only C_{18} precursors showed a similar trend at similar post-conceptional ages (4). These data collectively suggest a developing capacity for the infant to synthesize and accumulate erythrocyte AA with age. Although infants have been postulated to have a reduced capacity to synthesize LCPUFA from precursors such as LA and ALA, there has been little direct evidence except for differences in LCPUFA status between breast- and formula-fed infants at fixed postnatal ages. Our data suggest that there is indeed a limited capacity of the infant to produce AA, which is most pronounced in the first weeks after birth, but that by four months of age the formula-fed infant may have sufficient capacity to match AA levels of breast-fed infants from precursors. If this is established for all LCPUFA by metabolic studies (19,21), it will provide direct biochemical evidence in support of a conditional essentiality of LCPUFA in early post-natal life. If such a temporal essentiality is established, it may mean that infant formulas containing only LA and ALA can never be nutritionally complete regardless of the ratio of fatty acid substrates (22,23). Finally, it is interesting to speculate that variations in AA status between breast- and formula-fed infants may be partly explained by the timing of these trials (22,24,25).

The aim of adding EPO (containing GLA) to the supplemented formula was to provide a metabolic precursor of AA in an attempt to avoid the reduction in AA status associated with FO feeding (4,5). However, EPO treatment did not appear to maintain AA status as the proportion of erythrocyte AA in the FO + EPO-supplemented formula infants at 16 wk was similar to those of preterm infant fed FO formula (without EPO) at their equivalent follow-up (57 wk post-conceptional age) (5). So, despite the possible metabolic mechanisms working for the conservation of erythrocyte AA status, the addition of GLA did not significantly influence its maintenance in the presence of FO.

Our results show that GLA does not accumulate in erythrocyte membranes (Table 1). DGLA was the only n-6 LCPUFA in FO + EPO-supplemented infants not to decline below levels observed in either breast- or placebo formula-fed infants. Other studies that have used comparable daily doses of GLA (in the form of either EPO or blackcurrant seed oil) to supplement the diets of nursing mothers have demonstrated increases in both GLA and DGLA of total plasma and breast milk lipids (26). This study (26) was done in the absence of FO, but there are other reports that FO feeding in adults results in a reduction of DGLA status (27), although the FO dose used was 20-fold greater than that fed to the FO + EPO-supplemented infants. If FO, even in small doses, is inhibitory to the accumulation of DGLA, then it would appear that supplementation with GLA was as able to reverse this reduction.

At six weeks, the increase in LA observed in all dietary groups could be accounted for (within 1% of total fatty acids) by the changes observed in all 20- and 22-C PUFA. Although competition between LA and n-3 LCPUFA for incorporation into membranes is widely accepted and documented in both adult (28) and infant studies (7,29), interactions between LA and n-6 LCPUFA are less well investigated. Data from this study suggest that 22% of the variance in erythrocyte AA at six weeks can be attributed to the level of erythrocyte LA. It is therefore tempting to speculate that part of the reduction in AA levels was related to the early rise in LA that also occurred during this time. Although such inferences are difficult to make because both erythrocyte LA and AA levels may be affected by a multitude of other fatty acids, studies comparing the erythrocyte fatty acid status of breast- and standard formula-fed infants also have reported paradoxical reductions in AA levels in the face of high LA (reviewed in Ref. 22).

To summarize, the most pertinent changes observed in erythrocyte fatty acid status were in DHA, EPA, AA, and LA, as well as the interactions between them. DHA in breast and FO + EPO supplemented formula infants were maintained near or above day 5 levels for the 30-wk study period, whereas erythrocyte DHA in placebo formula-fed infants halved by week 16. The synthesis of DHA from ALA in placebo formula-fed infants is probably impeded by the presence of two rate-limiting δ6 desaturase steps in the metabolic pathway, as well as competitive inhibition by the large amount of LA, which is also a substrate for the δ6 desaturase enzyme(s). AA decreased in all infants in the first six weeks of life, and this reduction was most pronounced in FO + EPO-supplemented formula-fed infants. However, erythrocyte AA was similar to day 5 levels by week 30 in both the breast- and placebo formula-fed groups, suggesting a developing capacity to synthesize and incorporate AA. On the other hand, AA in FO + EPO-supplemented formula-fed infants remained at the six-week level, and part of this observation may be accounted for by the presence of EPA and DHA in the supplemented formula, which is a well-known competitor of AA for incorporation into membranes.

ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support of the Channel 7 Children's Medical Research Foundation, Nestle Australia, and Scotia Pharmaceutical, as well as the technical assistance of Ela Zielinski.

REFERENCES

1. Birch, D.G., Birch, E.E., Hoffman, D.R., and Uauy, R.D. (1992) Retinal Development in Very Low-Birth-Weight Infants Fed Diets Differing in Omega-3 Fatty Acids, *Invest. Ophthalmol. Vis. Sci. 33*, 2365–2376.
2. Birch, E.E., Birch, D.G., Hoffman, D.R., and Uauy, R. (1992) Dietary Essential Fatty Acid Supply and Visual Acuity Development, *Invest. Ophthalmol. Vis. Sci. 32*, 3245–3253.
3. Carlson, S.E., Werkman, S.H., Rhodes, P.G., and Tolley, E.A. (1993) Visual-Acuity Development in Healthy Preterm Infants: Effect of Marine-Oil Supplementation, *Am. J. Clin. Nutr. 58*, 35–42.
4. Carlson, S.E., Cooke, R.J., Rhodes, P.G., Peeples, J.M., Werkman, S.H., and Tolley, E.A. (1991) Long-Term Feeding of Formulas High in Linolenic Acid and Marine Oil to Very Low Birth Weight Infants: Phospholipid Fatty Acids, *Pediatr. Res. 30*, 404–412.
5. Hoffman, D.R. and Uauy, R. (1992) Essentiality of Dietary Omega-3 Fatty Acids for Premature Infants: Plasma and Red Blood Cell Fatty Acid Composition, *Lipids 27*, 886–895.
6. Carlson, S.E., Cooke, R.J., Werkman, S.H., and Tolley, E.A. (1992) First Year Growth of Preterm Infants Fed Standard Compared to Marine Oil n-3 Supplemented Formula, *Lipids 27*, 901–907.
7. Makrides, M., Simmer, K., Goggin, M., and Gibson, R.A. (1993) Erythrocyte Docosahexaenoic Acid Correlates with the Visual Response of Healthy, Term Infants, Pediatr. Res. 33, 425–427.
8. Birch, E., Birch, D., Hoffman, D., Hale, L., Everett, M., and Uauy, R. (1993) Breast-Feeding and Optimal Visual Development, *J. Pediatr. Ophthalmol. Strabismus 30*, 33–38.
9. Jorgensen, M.H., Jonsbo, F., Holmer, G., Hernell, O., and Michaelsen, K.F. 91994) Breast-Fed (BF) Term Infants Have a Better Visual Acuity than Formula Fed (FF) Infants at the Age of 2 and 4 Mo, *FASEB J. 8*, 460 (abstract).
10. Makrides, M., Neumann, M., Simmer, K., Pater, J., and Gibson, R. (1995) Are Long-Chain Polyunsaturated Fatty Acids Essential Nutrients in Infancy? *Lancet 345*, 1463–1468.
11. Nutrition statements adopted by the National Health and Medical Research Council 90th session in October 1980, *J. Food Nutr. 38*, 11–12.
12. Jackson, K.A., and Gibson, R.A. (1989) Weaning Foods Cannot Replace Breast Milk as Sources of Long-Chain Polyunsaturated Fatty Acids, *Am. J. Clin. Nutr. 50*, 980–982.
13. Makrides, M., Neumann, M.A., Byard, R.W., Simmer, K., and Gibson, R.A. (1994) Fatty Acid Composition of Brain, Retina, and Erythrocytes in Breast- and Formula-Fed Infants, *Am. J. Clin. Nutr. 60*, 189–194.
14. Norusis, M.J. (1993) Repeated Measures Analysis of Variance, in *SPSS for Windows: Advances Statistics, Release 6.0*, pp. 107–144, SPSS Inc., Chicago.
15. Makrides, M., Simmer, K., Neumann, M., and Gibson, R. (1995) Changes in the Polyunsaturated Fatty Acids of Breast Milk from Mothers of Full-Term Infants over 30 wk of Lactation, *Am. J. Clin. Nutr. 61*, 1231–1233.
16. Koletzko, B., Thiel, I., and Abiodun, P.O. (1992) The Fatty Acid Composition of Human Milk in Europe and Africa, *J. Pediatr. 120*, S62–S70.
17. Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H. (1991) The Metabolism of 7,10,13,16,19-Docosapentaenoic Acid to 4,7,10,13,16,19-Docosahexaenoic Acid in Rat Liver is Independent of a 4-Desaturase, *J. Biol. Chem. 266*, 19995–20000.
18. Sprecher, H. (1992) Interconversions Between 20- and 22-Carbon n-3 and n-6 Fatty Acids *via* 4-Desaturase Independent Pathways, in *Essential Fatty Acids and Eicosanoids: Invited Papers from the Third International Congress* (Sinclair, A.J. and Gibson, R.A. eds.) pp. 18–22, American Oil Chemists' Society, Champaign.
19. Emken, E.A., Adlof, R.O., and Gulley, R.M. (1994) Dietary Linoleic Acid Influences Desaturation and Acylation of Deuterium-Labeled Linoleic and Linolenic Acids in Young Adult Males, *Biochim. Biophys. Acta 1213*, 277–288.
20. Mantzioris, E., James, M.J., Gibson, R.A., and Cleland, L.G. (1994) Dietary Substitution with an α-Linolenic Acid-Rich Vegetable Oil Increases Eicosapentaenoic Acid Concentrations in Tissues, *Am. J. Clin. Nutr. 59*, 1304–1309.
21. Demmelmair, H., v. Schenck, Y., Behrendt, E., Sauerwald, T., and Koletzko, B. (1995) Estimation of Arachidonic Acid Synthesis in Full Term Neonates Using Natural Variation of ^{13}C-Abundance, *J. Pediatr. Gastroenterol. Nutr.*, in press.
22. Clark, K.J., Makrides, M., Neumann, M.A., and Gibson R.A. (1992) Determination of the Optimal Ratio of Linoleic Acid to α-Linolenic Acid in Infant Formulas, *J. Pediatr. 120*, S151–S158.
23. Gibson, R.A., Makrides, M., Neumann, M.A., Simmer, K. Mantzioris, E., and James, M.J. (1994) Ratios of Linoleic Acid to Linoleic Acid to α-Linolenic Acid in Formulas for Term Infants, *J. Pediatr. 125*, S48–S55.
24. Innis, S.M., Foote, K.D., MacKinnon, M.J., and King, D.J. (1990) Plasma and Red Blood Cell Fatty Acids of Low-Birth-Weight Infants Fed their Mother's Expressed Breast Milk or Preterm-Infant Formula, *Am. J. Clin. Nutr. 51*, 994–1000.
25. Putnam, J.C., Carlson, S.E., DeVoe, P.W., and Barness, L.A. (1982) The Effect of Variations in Dietary Fatty Acids on the Fatty Acid Composition of Erythrocyte Phosphatidylcholine and Phosphatidylethanolamine in Human Infants, *Am. J. Clin. Nutr. 36*, 106–114.

26. Gibson, R.A. and Rassias, G. (1990) Infant Nutrition and Human Milk, in *Omega-6 Essential Fatty Acids: Pathophysiology and Roles in Clinical Medicine* (Horrobin, D.F., ed.) pp. 283–293, Alan R. Liss, Inc., New York.
27. Cleland, L.G., Gibson, R.A., Neumann, M., and French, J.K. (1990) The Effect of Dietary Fish Oil Supplement upon the Content of Dihomo-Gammalinolenic Acid in Human Plasma Phospholipids, *Prostaglandins Leukot. Essent. Fatty Acids 40*, 9–12.
28. Cleland, L.G., James, M.J., Neumann, M.A., D'Angelo, M., and Gibson, R.A. (1992) Linoleate Inhibits EPA Incorporation from Dietary Fish-Oil Supplements in Human Subjects, *Am. J. Clin. Nutr. 55*, 395–399.
29. Clandinin, M.T., Parrott, A., Van Aerde, J.E., Hervada, A.R., and Lien, E. (1992) Feeding Preterm Infants a Formula Containing C_{20} and C_{22} Fatty Acids Composition of Infants Fed Human Milk, *Early Hum. Dev. 31*, 41–51.

[Received February 13, 1995, and in final revised form August 22, 1995; Revision accepted August 30, 1995]

Differences in Fatty Acid Composition of Immature and Mature Articular Cartilage in Humans and Sheep

Katherine A. Cleland[a], Michael J. James[a], Mark A. Neumann[b], Robert A. Gibson[b] and Leslie G. Cleland[a,*]

[a]Rheumatology Unit, Royal Adelaide Hospital, Adelaide, South Australia 5000, Australia and [b]Department of Pediatrics and Child Health, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia

ABSTRACT: Chondrocytes are imbedded in an avascular, highly charged extracellular matrix which could form a barrier to the transfer of dietary essential fatty acids (EFA) to chondrocytes. A study was designed to assess the composition of immature and mature joint cartilage with respect to essential and nonessential fatty acids relevant to EFA deficiency. Cartilage and muscle samples were obtained from human fetus, infant and adult cadavers, and from fetal and mature sheep. Lipid extracts were prepared and the fatty acid composition determined. In human and sheep joint cartilage, linoleic acid (LA; 18:2n-6) content was lower, and n-9 eicosatrienoic acid (ETrA; 20:3n-9) and arachidonic acid (AA; 20:4n-6) were higher in fetuses compared to mature subjects. An intermediate pattern was seen in infant cartilage. n-3 Fatty acids tended to be higher in fetal than in mature cartilage in humans and in sheep. In human muscle (and in other noncartilaginous comparison tissues), similar differences between fetuses and adults were seen in LA and AA, but not in ETrA. In fetal sheep muscle, very low LA, reduced AA and raised ETrA levels compared to mature sheep muscle were seen. However, although the pattern is characteristic of EFA deficiency, the abundance of n-6 EFA in liver and spleen of human fetuses and of n-3 EFA in liver and spleen of fetal sheep suggests that placental transfer of EFA is not likely to be limiting. During fetal development, the metabolism of fatty acids is distinctive and differs between the species. ETrA appears to be a readily measurable component of some tissues at certain stages of development when its presence in tissues does not indicate EFA deficiency.

Lipids 30, 949–953 91995).

The 20-carbon n-6 fatty acid, arachidonic acid (AA; 20:4n-6), is an important component of structural phospholipids and is a precursor for the eicosanoid mediators of cell metabolism, platelet-vascular homeostasis, and inflammation. However, mammals are unable to introduce double bonds into the n-6 position of long-chain fatty acids, and the essential cellular requirements for AA are normally met by ingestion of its 18-carbon precursor linoleic acid (LA; 18:2n-6) (reviewed in Ref. 1). Once ingested, LA is desaturated and elongated to AA. Thus, LA is a dietary essential fatty acid (EFA). By contrast, the 18-carbon n-9 fatty acid, oleic acid (OA; 18:1n-9), can be synthesized endogenously (2). In the presence of dietary sufficiency of LA, the desaturation and elongation of OA to the 20-carbon n-9 fatty acid, eicosatrienoic acid (ETrA; 20:3n-9), are inhibited by substrate competition between OA and LA. However, if tissues are deprived of LA, either through dietary insufficiency or diminished uptake of LA, then the level of ETrA increases (3). For this reason, the ratio of ETrA to AA in plasma has been used as a biochemical marker for EFA deficiency, which is associated with accumulation of ETrA and depletion of AA and LA (4). n-3 Fatty acids also are essential dietary components, and their presence in tissues can be used as an index of EFA.

ETrA is normally either undetectable or found at very low levels in tissues and plasma. Recently, Adkisson and coworkers (5) reported that immature hyaline cartilage in humans and in certain other vertebrates (6) has a fatty acid profile similar to that seen in other tissues in EFA deficiency; i.e., they reported that cartilage has relatively high levels of ETrA and low levels of n-6 fatty acids. These authors argued that, because cartilage is avascular with a highly charged extracellular matrix, chondrocytes may be deprived of n-6 EFAs relative to cells in other tissues. n-6 Eicosanoids have been shown to have important effects on the metabolism of chondrocytes cultured *in vitro* (7). The level of eicosanoid precursor, AA, within chondrocytes, and the level of ETrA, which can inhibit the metabolism of M to eicosanoids (8), will be critical determinants of the extent of eicosanoid synthesis by chondrocytes. Furthermore, the level of these fatty acids also may influence the effects on cartilage of antiarthritic drugs which can inhibit eicosanoid synthesis (9). The present study was designed to analyze the fatty acid composition of human and sheep articular cartilage at different stages of maturity.

METHODS

Human cadaver specimens. Cartilage was taken from cadavers of fetuses (death *in utero*), infants [sudden infant death

*To whom correspondence should be addressed at Rheumatology Unit, Royal Adelaide Hospital, North Terrace, Adelaide, SA 5000, Australia.

Abbreviations: AA, arachidonic acid (20:4n-6); EFA, essential fatty acid, ETrA, eicosatrienoic acid (20:3n-9); LA, linoleic acid (18:2n-6); OA, oleic acid (18:1n-9); SIDS, sudden infant death syndrome.

Copyright © 1995 by AOCS Press

syndrome (SIDS)], and adults without a known history of inflammatory disease. Ten nonmacerated fetuses were studied; mean duration of gestation 25 wk, range 18–38 wk. Six SIDS cadavers were studied; mean age 6 mon, range 1–8 mon. Details of the fourteen adult cadavers studied are shown in Table 1. In fetuses and infants, the entire femoral head was excised and stored at −70°C prior to extraction of lipids from the sample. Full thickness cartilage specimens were obtained as shavings from the articular surfaces of 13 knees (femoral condyles and tibial plateau) from 13 adults and of 10 shoulders (glenoid cavity and head of humerus) from 10 adults. In joints where there was evidence of degenerative change, cartilage was taken from both macroscopically degenerate and macroscopically normal cartilage, which were processed and assayed separately. Skeletal muscle samples were taken from psoas major or quadriceps muscle. Liver and spleen samples were also taken in adults and fetuses.

Sheep specimens. Cartilage (elbow and stifle joints) and skeletal muscle samples were obtained from sheep fetuses and nonarthritic mature wethers shortly after slaughter. Liver and spleen samples from two of the fetal sheep also were analyzed.

Determination of arthritic status of joints. Knee and shoulder joints of adult cadavers were examined macroscopically for evidence of osteoarthritis and graded for severity (10). Initially, India-ink staining also was used to highlight osteoarthritic areas as the ink adheres to areas of cartilage fibrillation but not to healthy cartilage.

Fatty acid analyses. Cartilage shavings from knees or shoulders of adults or from the femoral head of fetuses or infant cadavers were rinsed thoroughly in normal saline to remove adherent body fluids. The tissues were then homogenized using a power homogenizer with a rotating blade (Ultra-Tarrax Type TP18/10; Janke-Kunkel, Staufen, Germany).

TABLE 1
Clinical Details of Cadavers

Age (yr)	Sex	Principle diagnoses	Status of joints[a]
37	F	Craniopharyngioma	+
37	M	Alcoholic liver disease, bleeding varices	–
37	F	Non-Hodgkins lymphoma, thymoma	–
57	F	Myocardial infarction	+
67	M	Intracerebral hemorrhage	+
72	F	Myocardial infarction	++
75	M	Multisystems failure, alcoholic liver disease, prostatic carcinoma	++,–
76	F	Carcinomatosis	++
78	F	Multisystems failure, chronic liver disease	+++
79	M	Ischemic cardiomyopathy	+
82	F	Pneumonia, gastric ulcer	+++
85	F	Subarachnoid hemorrhage	++,
87	M	Cholelithiasis, septicemia	–
90	M	Pituitary tumor	+

[a]+,–, Arthritic and nonarthritic joints sampled; –, no degeneration; +, evidence of minor degenerative changes; ++, more advanced degenerative change in some areas, no full thickness cartilage loss; and +++, advanced degenerative disease with full thickness cartilage loss in some areas. In arthritic joints, samples were taken from macroscopically arthritic and normal regions and analyzed separately for fatty acid composition.

Lipids were extracted from tissue with chloroform/methanol, and extracts were evaporated to dryness under nitrogen. The samples were methylated in 1% H_2SO_4 in methanol at 70°C for three hours. When cooled, the resulting methyl esters were extracted into *n*-heptane and transferred to vials containing anhydrous Na_2SO_4 as the dehydrating agent. Fatty acid methyl esters were separated and quantified using a Hewlett-Packard (Avondale, PA) 5880 gas chromatograph equipped with a 50-m capillary column (0.33 mm i.d.) coated with BPX-70 (0.25 μm film thickness; SGE Pty. Ltd., Victoria, Australia). The injector temperature was set at 250°C and the detector (flame-ionization) temperature at 300°C. The initial oven temperature was 140°C and was programmed to rise to 220°C at 5°C/min. Helium was used as the carrier gas at a velocity of 35 cm/s. Fatty acid methyl esters were identified based on the retention time relative to authentic lipid standards obtained from Nu-Chek-Prep Inc. (Elysian, MN). Standards included saturated fatty acids C_6–C_{24} and unsaturated fatty acids $C_{14:1}$–$C_{22:6}$. Correction factors were not required for any of the fatty acids detected in the tissue samples. Reproducibility (coefficient of variation) was <1% for the major peaks (>5% total fatty acids) and <5% for minor peaks.

Statistics. The difference in fatty acid composition of the sample groups was examined by Student's *t*-test.

RESULTS

Human samples (Tables 2–4). Levels of fatty acids in adult cartilage were not significantly different between arthritic and nonarthritic joints and between arthritic and nonarthritic areas of cartilage from arthritic joints. Data obtained from different cartilage samples of individual joints were, therefore, pooled to yield mean values for individual joints, which have been used in comparisons between mature and immature articular cartilage.

There were significant differences ($P < 0.05$) between human adult and human fetal cadavers with regard to levels of LA and AA found in all of the tissues tested, i.e., cartilage, muscle, liver, and spleen. LA levels were higher in adults and, by contrast, AA levels were higher in fetuses. In infant cartilage, LA and AA levels were intermediate between those found in fetuses and adults. The levels of LA and AA in muscle were similar in infants and in adults.

A possible late age-related increase in AA as a percentage of fatty acids in cartilage was suggested by a comparison of the younger adult cadavers (6.4 ± 1.3, n = 3, age less than 40 yr) with those of advanced age (10.2 ± 2. 1, n = 10, age greater than 65 yr, $P = 0.015$). AA levels were similar in the muscle samples of the younger and older cadavers, and no differences were seen in the proportions of the other unsaturated fatty acids in muscle or cartilage (data not shown). No significant differences were found on comparison of fatty acids in cartilage samples from shoulders and knees (data not shown).

Mean ETrA was 2.0% of fatty acids in fetal cartilage, 1.5% in infant cartilage, and below the limit of detection (<0.1%) in adult articular cartilage ($P < 0.001$). There were no signifi-

cant differences between fetal and infant ETrA levels, which were both significantly different from adult levels. Differences in ETrA between adult and fetus were not significant in the other tissues studied.

In a similar manner to variations in AA, the levels of ETrA in cartilage varied inversely with levels of LA. This did not occur in muscle where the level of ETrA was not elevated in fetal or infant tissue. Despite the large variation in fetal, infant, and adult cartilage fatty acids, the aggregate amount of LA, AA, and ETrA was similar (range 18–20% of fatty acids). Total amounts of muscle LA, AA, and ETrA were greater in infants and adults (38–39%) than in fetal tissue (25%).

n-3 Fatty acids were higher in fetal cartilage than in infant or adult cartilage ($P < 0.01$), which were essentially similar in this respect. n-3 Fatty acids were found at similar levels in fetal and adult liver and were higher in adult than in fetal spleen ($P < 0.01$). Docosahexaenoic acid was the dominant n-3 fatty acid in all human tissues sampled.

Sheep samples (Table 5). ETrA was significantly higher in fetal cartilage and muscle, compared with adult tissues ($P < 0.001$). LA was significantly lower in fetal cartilage and muscle, compared with adult tissues ($P < 0.001$). AA was significantly higher in fetal, compared with adult cartilage ($P < 0.01$), but was significantly lower in fetal compared with adult muscle ($P < 0.01$). As seen in humans, the total amount of LA, AA, and ETrA was similar for fetal and mature sheep in cartilage (9–10% fatty acids), but not in muscle (fetuses 9.4%, wethers 27%). Total n-3 fatty acids were slightly higher in fetal than in mature cartilage ($P < 0.05$) and lower in the muscle of fetuses than in withers. Substantial amounts of n-3 fatty acids were found in fetal liver and spleen (18.8 and 12.6% of fatty acids, respectively). Thus, the overall pattern of fatty acids in fetal sheep tissues did not suggest EFA deprivation.

Relevant trace or minority fatty acids. In two of the six infant cartilage samples, 18:2n-9 was detected with levels of 1.9 and 3.1%. This fatty acid was not detected in any of the other infant cartilage samples, nor in any of the other human or sheep material analyzed. Small amounts of 22:3n-9 were found in various samples, but always less than 1% of total fatty acids. No 18:3n-9 was detected in any sample. Small amounts of 20:2n-6 were resolved, but were always less than 1% of fatty acids and typically less than 0.4%. No 18:3n-3 was detected in cartilage, and only trace amounts were found in other tissues (<0.2%).

TABLE 2
Fatty Acid Composition of Femoral Head from Human Fetus and Infant and Adult Articular Cartilage

	Fatty acids (% total fatty acids)[a]		
	Fetal cartilage (n = 10)	Infant cartilage (n = 6)	Adult cartilage (n = 14)
16:0	22.1 ± 1.7	21.1 ± 1.2	33.6 ± 7.4[c,d]
18:0	14.5 ± 1.1	17.7 ± 2.7	16.5 ± 2.5
Total saturates[b]	42.9 ± 2.2	45.2 ± 3.6	55.1 ± 3.6[c,d]
16:1n-7	1.4 ± 0.5	0.4 ± 0.5[e]	0.6 ± 1.3
18:1n-7	3.8 ± 0.5	3.2 ± 0.5	2.6 ± 0.9[d]
18:1n-9	21.4 ± 3.4	22.3 ± 0.8	13.5 ± 1.7[c,d]
24:1n-9	0.3 ± 0.2	0.4 ± 0.4	3.4 ± 1.5[c,d]
Total monosaturates	27.6 ± 4.8	27.5 ± 2.3	20.3 ± 2.1[c,d]
20:3n-9	2.0 ± 0.6	1.5 ± 0.9[e]	<0.1[c,d]
18:2n-6	2.9 ± 0.8	6.1 ± 1.0[e]	8.9 ± 1.8[d]
20:3n-6	1.5 ± 0.3	1.6 ± 0.3	1.0 ± 0.4[c,d]
20:4n-6	15.2 ± 2.8	11.0 ± 1.5[e]	9.1 ± 2.5[d]
Total n-6	24.0 ± 3.9	23.5 ± 3.2	20.0 ± 4.1
20:5n-3	<0.1	<0.1	<0.1
22:5n-3	0.1 ± 0.2	0.4 ± 0.2	0.4 ± 0.7
22:6n-3	2.8 ± 1.2	1.1 ± 0.6	0.8 ± 0.6
Total n-3	2.9 ± 1.3	1.5 ± 0.5[d]	1.3 ± 1.3[d]

[a]Mean ± SD. [b]Includes dimethyl acetal fatty acids. [c]Adult vs. infant, $P < 0.01$. [d]Adult vs. fetus, $P < 0.01$. [e]Infant vs. fetus, $P < 0.01$.

TABLE 3
Fatty Acid Composition of Muscle from Human Fetus, Infant, and Adult

	Fatty acids (% total fatty acids)[a]		
	Fetal muscle (n = 10)	Infant muscle (n = 5)	Adult muscle (n = 12)
16:0	18.8 ± 0.6	15.7 ± 2.1	16.0 ± 1.6
18:0	13.9 ± 1.4	16.5 ± 1.4	13.3 ± 1.3[c]
Total saturates[b]	41.1 ± 1.5	40.7 ± 1.2	39.9 ± 2.1
16:1n-7	0.6 ± 0.3	0.3 ± 0.1	1.1 ± 0.8
18:1n-7	3.1 ± 0.5	1.8 ± 0.6[e]	2.4 ± 0.7
18:1n-9	19.0 ± 5.8	9.3 ± 1.6[e]	9.7 ± 2.0[d]
24:1n-9	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
Total monosaturates	23.3 ± 6.1	12.1 ± 2.2[e]	14.0 ± 3.1[d]
20:3n-9	0.2 ± 0.2	0.2 ± 0.1	0.3 ± 0.2
18:2n-6	8.2 ± 5.4	25.5 ± 0.3	24.9 ± 4.6
20:3n-6	1.9 ± 1.1	1.9 ± 0.4	1.2 ± 0.2[c]
20:4n-6	17.0 ± 1.7	13.2 ± 1.3	12.0 ± 1.8[d]
Total n-6	32.6 ± 4.9	43.1 ± 1.7[e]	40.5 ± 4.0[d]
20:5n-3	<0.1	0.3 ± 0.2	0.7 ± 0.3
22:5n-3	0.5 ± 0.3	1.6 ± 0.9	1.9 ± 0.7
22:6n-3	2.1 ± 0.4	2.0 ± 0.3	2.3 ± 1.2
Total n-3	2.6 ± 0.6	3.9 ± 0.7[e]	5.0 ± 2.3[d]

[a]Mean ± SD. [b]Includes dimethyl acetal fatty acids. [c]Adult vs. infant, $P < 0.01$. [d]Adult vs. fetus, $P < 0.01$. [e]Infant vs fetus, $P < 0.01$.

DISCUSSION

In humans, differences in fatty acid composition between fetuses and adults were found in all tissues studied. Although fetal LA levels were lower in all tissues and cartilage ETrA was higher in the fetus than in the adult, the picture differed

TABLE 4
Fatty Acid Composition of Liver and Spleen from Human Fetus and Adult

	Fatty acids (% in total fatty acids)[a]			
	Fetal liver (n = 10)	Adult liver (n = 10)	Fetal spleen (n = 10)	Adult spleen (n = 12)
16:0	22.2 ± 2.0	26.1 ± 4.1	19.9 ± 1.8	22.6 ± 2.6
18:0	16.8 ± 1.4	17.5 ± 2.7	16.8 ± 1.3	14.4 ± 2.1
Total saturates[b]	41.0 ± 2.4	45.8 ± 5.3	43.7 ± 2.9	44.3 ± 3.6
16:1n-7	1.3 ± 0.6	1.5 ± 0.8	0.5 ± 0.3	0.9 ± 0.5
18:1n-7	3.0 ± 0.5	2.3 ± 0.4[c]	2.7 ± 0.5	2.9 ± 1.0
18:1n-9	11.9 ± 2.7	12.9 ± 2.7	13.5 ± 2.9	14.1 ± 2.9
24:1n-9	0.4 ± 0.1	0.1 ± 0.1[c]	0.7 ± 0.1	0.5 ± 0.3
Total monosaturates	16.9 ± 3.5	17.2 ± 3.6	18.0 ± 3.5	19.4 ± 3.9
20:3n-9	0.4 ± 0.2	0.2 ± 0.3	0.1 ± 0.2	0.2 ± 0.2
18:2n-6	6.6 ± 1.6	14.6 ± 4.1[c]	3.7 ± 1.0	6.1 ± 1.6[c]
20:3n-6	2.3 ± 0.5	1.5 ± 0.7[c]	1.9 ± 0.3	1.3 ± 0.3[c]
20:4n-6	22.9 ± 3.2	12.2 ± 1.9[c]	21.3 ± 1.5	16.7 ± 2.2[c]
Total n-6	33.9 ± 2.8	29.3 ± 4.7[c]	33.4 ± 2.8	29.1 ± 3.5[c]
20:5n-3	0.2 ± 0.2	0.8 ± 1.1	<0.1	0.3 ± 0.3
22:5n-3	0.3 ± 0.2	1.1 ± 0.4[c]	0.6 ± 0.2	3.0 ± 0.8[c]
22:6n-3	7.3 ± 2.2	2.4 ± 1.2[c]	4.2 ± 1.2	3.6 ± 0.9
Total n-3	7.8 ± 2.3	7.5 ± 3.8	4.8 ± 1.6	7.0 ± 1.7[c]

[a]Mean ± SD. [b]Includes dimethyl acetal fatty acids. [c]Adult vs. fetus, $P < 0.01$.

from EFA deficiency in that the AA levels in all human fetal tissues sampled were significantly higher than in their adult counterparts. Furthermore, eicosapentaenoic acid levels were higher in fetal than in infant and in adult cartilage. The findings suggest a general alteration in EFA utilization in the fetus compared to adults rather than a cartilage-specific barrier to the transfer of EFA.

In fetal lamb cartilage, LA was lower but AA, ETrA, and total n-3 fatty acids were higher than in adult cartilage. In fetal lamb muscle, both LA and AA were lower and ETrA higher compared to mature sheep. This pattern in muscle, at least, more closely resembles EFA deficiency. However, the abundance of AA and n-3 fatty acids in fetal liver and spleen argues strongly against a state of EFA deprivation in sheep fetuses.

The observed differences between fetal and mature tissues in humans and sheep show that tissue fatty acid composition data obtained in fetal or immature animals cannot be taken as necessarily representative of the composition of their mature counterparts. The study of fatty acid composition in hyaline cartilage of ten-week-old chickens, fetal calves, and newborn pigs reported by Adkisson and co-workers (5) needs to be interpreted with this reservation in mind. In a very recent paper, Adkisson and co-workers have documented changes in fatty acid composition of articular cartilage of chicks fed test diets from age one-day to eight weeks (11). They observed changes in the content of saturated and unsaturated fatty acids (including EFA) within cartilage, which was influenced by the relative amounts of relevant fatty acids given in the diet. These latter findings, together with the present study, suggest that chondrocytes are not deprived of access to EFA because of their charged extracellular matrix, but rather that cartilage (like other tissues) has its own distinct pattern of fatty acid metabolism, of which ETrA accumulation, at certain stages of development is a manifestation.

The lower LA seen in the fetus may reflect, at least in part, different utilization of n-6 fatty acids during fetal life such that AA and ETrA accumulation is favored at the expense of LA. Compared to corresponding fetal samples, human infant cartilage and muscle exhibited substantially higher LA, which was similar to levels found in adult samples. Furthermore, LA content in infant cadavers appeared to be proportional to the age of the infant at the time of death (data not shown) although the size of the sample was too small to adequately assess the significance of difference in LA between infants. Notwithstanding, the change toward the adult pattern appears to occur soon after birth, suggesting that a change in LA metabolism occurs in early infancy.

Certain differences in fatty acid metabolism between fetuses and adults may be tissue-specific. Because femoral head specimens, which contain both articular cartilage and unossifed enchondral bone, were taken from both fetuses and infants, a putative metabolic difference between articular cartilage and enchondral bone could not fully explain the observed differences in fatty acid composition between fetal and infant femoral head cartilage. Furthermore, the fetal pattern of low LA and high AA relative to adult samples also was seen in liver and spleen.

In our analysis of material from cadavers without a history of clinically important arthritic disease during life, the presence of cartilage degeneration did not appear to influence the fatty acid composition of articular cartilage. However, it should be noted that one or more of the sampled joints of most subjects exhibited macroscopic and microscopic degenerative changes, and few entirely normal joints were studied, even in the younger adults. Notwithstanding, AA levels were lower in the younger adult cadavers than in those of advanced age. The weight-bearing status of the joints sampled (shoulder vs. knee) did not appear to influence their fatty acid composition.

For human articular cartilage, higher values of ETrA than AA and very low proportions of LA in "rapidly growing cartilage" have been reported (6). We have been unable to confirm this described dominance of ETrA over AA in either human or sheep fetus or in human infant hyaline cartilage, in which we have found AA to be increased compared to adult levels. The lower LA and raised ETrA in fetal compared to adult joint cartilage have been confirmed. It also was reported that ETrA was depleted in weight-bearing human articular cartilage after 25–30 yr (6). However, no comparison with nonweight-bearing joints, nor any data on the age of the younger subjects was provided, except for a statement that

TABLE 5
Fatty Acid Composition of Fetal and Mature Cartilage and Muscle and Fetal Liver and Spleen in Sheep

	Fatty acids (% total fatty acids)[a]					
	Fetal cartilage (n = 12)	Mature cartilage (n = 6)	Fetal muscle (n = 9)	Mature muscle (n = 3)	Fetal liver (n = 2)	Fetal spleen (n = 2)
16:0	20.1 ± 1.5	26.1 ± 7.8	15.8 ± 1.4	12.0 ± 0.4[c]	21.7 ± 1.0	20.2 ± 1.0
18:0	15.0 ± 1.4	15.9 ± 4.3	12.5 ± 1.1	8.8 ± 0.2[c]	16.6 ± 1.0	12.6 ± 1.3
Total saturates[b]	41.0 ± 1.7	50.1 ± 10.4	37.3 ± 1.9	36.6 ± 0.4	43.2 ± 2.3	41.8 ± 0.3
16:1n-7	1.4 ± 0.1	0.5 ± 0.7	1.5 ± 0.2	0.7 ± 0.1[c]	1.4 ± 0.1	0.9 ± 0.1
18:1n-7	4.9 ± 0.3	3.4 ± 1.2	5.0 ± 0.6	1.1 ± 0.1[c]	4.1 ± 0.5	4.4 ± 0.4
18:1n-9	30.4 ± 2.4	25.6 ± 8.0	35.5 ± 3.3	21.0 ± 1.0[c]	18.5 ± 0.3	24.9 ± 1.6
24:1n-9	0.3 ± 0.2	4.8 ± 3.6[c]	0.3 ± 0.2	0.4 ± 0.3	<0.1	<0.1
Total monosaturates	39.9 ± 3.1	34.9 ± 12.2	44.7 ± 3.6	25.0 ± 0.7[c]	26.6 ± 0.9	32.9 ± 1.6
20:3n-9	3.0 ± 0.5	0.1 ± 0.2[c]	4.3 ± 0.9	1.0 ± 0.3[c]	1.3 ± 0.1	1.8 ± 0
18:2n-6	0.6 ± 0.6	6.0 ± 1.8[c]	0.8 ± 0.2	15.9 ± 0.9[c]	1.3 ± 0	1.1 ± 0.1
20:3n-6	0.2 ± 0.2	<0.1	0.2 ± 0.1	0.8 ± 0[c]	0.5 ± 0	0.4 ± 0
20:4n-6	6.0 ± 1.2	4.1 ± 1.2	4.3 ± 1.2	10.5 ± 0.3[c]	7.7 ± 0.8	8.3 ± 1.1
Total n-6	7.8 ± 1.4	10.9 ± 3.4	6.4 ± 1.8	27.6 ± 0.5[c]	10.1 ± 1.6	10.6 ± 1.3
20:5n-3	0.9 ± 1.1	0.4 ± 0.6	1.0 ± 2.0	2.6 ± 0.2	2.3 ± 0.2	1.8 ± 0.4
22:5n-3	3.1 ± 1.0	2.6 ± 1.5	3.8 ± 1.1	3.2 ± 0.1	8.8 ± 0.1	7.6 ± 0.1
22:6n-3	2.7 ± 0.3	1.0 ± 0.8	2.0 ± 1.5	1.1 ± 0.2	7.7 ± 0.2	3.2 ± 0.3
Total n-3	6.6 ± 2.1	4.0 ± 2.5	6.8 ± 2.1	9.9 ± 0.6	18.8 ± 0.1	12.6 ± 0

[a]Mean ± SD. [b]Includes dimethyl acetal fatty acids. [c]Mature vs. fetus, $P < 0.01$.

the overall age range of subjects was 0–86 yr (6). In agreement with the present study, they found no difference in n-6 fatty acids in normal and osteoarthritic cadaver joints. By comparison, n-6 fatty acids in cartilage samples obtained at the time of joint replacement surgery were higher. The operative specimens are likely to contain significant amounts of fibrous tissue and fibrocartilage, which can replace hyaline cartilage in end-stage joint failure. Thus, the altered fatty acid composition in these samples could reflect the presence of tissue other than hyaline cartilage.

In summary, the fatty acid composition of articular cartilage does not suggest a tissue-specific reduced EFA state. The proportions of n-6 fatty acids in cartilage alter in association with maturation and aging, at least partly independently of joint degeneration.

REFERENCES

1. Simopoulos, A.P. (1991) Omega-3 Fatty Acids in Health and Disease and in Growth and Development, *Am. J. Clin. Nutr. 54*, 438–463.
2. Pudelkewicz, C., Seufert, J., and Holman, R.T. (1968) Requirements of the Female Rat for Linoleic and Linolenic Acids, *J. Nutr. 94*, 138–146.
3. Brenner, R.R., and Peluffo, R.O. (1966) Effect of Saturated and Unsaturated Fatty Acids on the Desaturation *in vitro* of Palmitic, Stearic, Oleic, Linoleic and Linolenic Acids, *J. Biol. Chem. 241*, 5213–5219.
4. Holman, R.T. (1977) Essential Fatty Acid Deficiency in Humans, in *Handbook of Nutrition and Foods* (Recheigle, M., ed.) CRC Press, Cleveland.
5. Adkisson, H.D., Risener, F.S., Zarrinkar, P.P., Walla, M.D., Christie, W.W., and Wuthier, R.E. (1991) Unique Fatty Acid Composition of Normal Cartilage: Discovery of High Levels of n-9 Eicosatrienoic Acid and Low Levels of n-6 Polyunsaturated Fatty Acids, *FASEB J. 5*, 344–353.
6. Adkisson, H.D., Trancik, T.M., Zarrinkar, P.P., and Wuthier, R.E. (1990) Accumulation of Essential Fatty Acids in Hyaline Cartilage with Aging and Joint Disease: Possible Implications in Development of Osteoarthritis, *Orthopaedic Trans. 14*, 423–424.
7. Kemick, M.L.S. , Chin, J.E., and Wuthier, R.E. (1989) Role of Prostaglandins in Differentiation of Growth Plate Chondrocytes, *Adv. Prostaglandin Thromboxone Leukotriene Res. 18*, 423–426.
8. Stenson, W.F., Prescott, S.M., and Sprecher, H. (1984) Leukotriene B Formation by Neutrophils from Essential Fatty Acid-Deficient Rats, *J. Biol. Chem. 259*, 11784–11789.
9. Mizuno, K., Yamamoto, S., and Lands, W.E.M. (1982) Effects of Nonsteroidal Anti-Inflammatory Drugs on Fatty Acid Cyclooxygenase and Prostaglandin Hydroperoxidase Activities, *Prostaglandins 23*, 734–757.
10. Collins, D.H. (1949) Osteoarthritis, in *The Pathology of Articular and Spinal Diseases*, pp. 76–81, Edward Arnold, London.
11. Xu, H., Watkins B.A., and Adkisson H.D. (1994) Dietary Lipids Modify the Fatty Acid Composition of Cartilage, Isolated Chondrocytes and Matrix Vesicles, *Lipids 29*, 619–625.

[Received August 29, 1994, and in final revised form August 11, 1995; Revision accepted August 31, 1995]

Dietary Fish Oil and Cytochrome P-450 Monooxygenase Activity in Rat Liver and Kidney

Elena Valdes, Patricio Vega, Nicolas Avalos and Myriam Orellana*

Departamento de Bioquimica, Facultad de Medicina, Universidad de Chile, Casilla 70086, Santiago 7, Chile

ABSTRACT: Lauric acid hydroxylation and aminopyrine *N*-demethylation were studied in kidney and liver microsomes from rats treated with fish oil. Different doses of fish oil containing 20% eicosapentaenoic acid and 10% docosahexaenoic acid were provided daily to the rats for seven days. In all the groups studied, the lauric acid metabolism was higher in kidney microsomes and the aminopyrine metabolism in the liver microsomes. Although no effect on the renal cytochrome P-450 concentration was detectable, all four fish oil doses increased the hepatic concentration of cytochrome P-450 by a mean 27%. The higher fish oil doses used increased the renal and hepatic microsomal metabolism of aminopyrine. The lauric acid metabolism was increased by fish oil only in the liver. Fish oil, a known inducer of fatty acid peroxisomal β-oxidation, also induced microsomal activity. These results show that liver and kidney respond in different ways to dietary factors such as fish oil. In addition, our study would suggest that fish oil increased the activity of two different families of liver cytochrome P-450. The activity of kidney lauric acid 11- and 12-hydroxylation, however, was not modulated by fish oil.

Lipids 30, 955–958 (1995).

Microsomal cytochrome P-450 catalyzes the oxidative metabolism of a wide variety of lipophilic compounds, such as drugs, carcinogens, endogenous steroids (1), and fatty acids (2). Cytochrome P-450s are found predominantly in the liver, but are also present in many other tissues, such as the kidney.

Marine oils are rich in polyunsaturated fatty acids of n-3 family, especially eicosapentaenoic acid (20:5) (EPA) and docosahexaenoic acid (22:6) (DHA). There are several cytochrome P-450 isoenzymes from the family 4A that oxidize fatty acids. The CYP4A1 purified from liver of clofibrate-treated rats catalyzes the ω-hydroxylation of arachidonic and lauric acids (3). The physiological role of these isoenzymes P-450 has not been identified.

It has been reported that clofibrate and other peroxisomal proliferators simultaneously enhance the cellular content and activity of liver cytochrome P-450 (3). In previous studies with starved rats, we showed an increase in the microsomal lauric acid ω-hydroxylation, which was correlated with an increase in peroxisomal fatty acid β-oxidation in liver and in kidney (4,5).

The liver microsomal activity has been studied extensively. Meanwhile, our knowledge of both the constitutive and inducible content and activity of kidney cytochrome P-450 remains very limited. The levels of cytochrome P-450 in kidney previously have been shown to be much lower than those seen in liver, and the renal cortex presents the highest levels of cytochrome P-450 (6,7).

It has been reported that fish oil or diets enriched in EPA and DHA induce liver fatty acid microsomal and peroxisomal oxidation (8–11). The extent of the increase of microsomal activity or peroxisomal β-oxidation depends on the type of dietary fish oil (10). However, the effect of fish oil on kidney microsomal activity has not been reported.

In this work, we study the effect of fish oil consumption on microsomal lauric acid hydroxylation and aminopyrine *N*-demethylation to investigate the possible induction of the microsomal activity by this factor. We carried out a comparative study using liver and kidney microsomes in order to determine how these tissues respond to a diet enriched with polyunsaturated fatty acids EPA and DHA.

MATERIALS AND METHODS

Chemicals. Fish oil was provided by Maver Laboratory (Santiago, Chile). According to the manufacturers, the fish oil was encapsulated under vacuum using anerobic conditions. The capsules were stored at room temperature upon arrival and opened just prior to the rat gavages. The fish oil was a sardine oil concentrate containing 36.5% polyunsaturated fatty acids [20.4% EPA, 10.3% DHA, 1.4% linoleic, 0.8% linolenic, 1.6% octadecatetraenoic (18:4n-3), 0.4% eicosadienoic (20:2n-9), 1.1% arachidonic, and 0.5% docosatetraenoic acids (22:4n-6)]; 32.8% monounsaturated fatty acids [12.4% palmitoleic, 16% oleic, 2.5% eicosanoic (20:1n-9), and 1.9% cetoleic acids], and 30.7% saturated fatty acids (0.7% pentadecanoic, 20.1% palmitic, 1.0% heptadecanoic, 4.1% tetracosanoic, and 4.8% stearic acids). The corn oil used in our control rats was provided by Mazola (Inducoen, Santiago, Chile), and contained 60% total polyunsaturated fatty acids (57.7% linoleic and 2.3% linolenic acids); 26.1% of the mo-

*To whom correspondence should be addressed at Departamento de Bioquimica, Facultad de Medicina, Universidad de Chile, Independencia 1027, Casilla 70086, Santiago 7, Chile.

Abbreviations: ANOVA, analysis of variance; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Copyright © 1995 by AOCS Press

nounsaturated oleic acid, and 13.6% saturated fatty acids (10.7% palmitic, 2.8% stearic, and 0.1% docosanoic acids). Lauric acid, aminopyrine, NADPH, isocitrate dehydrogenase, and sodium isocitrate were purchased from Sigma Chemical Co. (St. Louis, MO). (1-^{14}C] Lauric acid (56 mCi/mmol) was from Amersham (Arlington Heights, IL). All the other chemicals were obtained from commercial sources and were of the highest purity available.

Animals and treatments. Mature male Wistar rats (Charles River Breeding Laboratories, North Wilmington, MA) (weighing 200–250 g) were used throughout. Rats were fed for seven days with either pure corn oil or the fish oil described previously (0.5–7.5 g/kg body weight). The lowest fish oil dose used was comparable to that used clinically in Chile. The daily oil supplementation was given by gastric tube feeding, and an equivalent volume was applied to control and to treated rats (0.1–1.5 mL). All the animals had free access to standard pellets and water.

Microsomal activities. Microsomes from whole kidney or liver were prepared as described elsewhere (12). Decapsulated kidneys or livers of 3–5 rats were used for each microsomal sample. The total cytochrome P-450 content was measured as described by Omura and Sato (13). Sodium succinate was added to kidney microsome preparations to minimize hemoprotein interferences in light transmission, which become significant in tissues with low cytochrome P-450 content (14).

Lauric acid hydroxylation. This activity measurement was performed according to the method described previously (4). Lauric acid and its 11-hydroxy and 12-hydroxy derivatives were resolved by a reverse-phase high-performance liquid chromatographic technique as previously described (5).

Aminopyrine N-demethylation. The measurement of oxidative demethylation was performed according to the method described by Nash (15). The reaction mixture contained 10 mM aminopyrine, 50 mM buffer Tris–Cl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 8 mM sodium isocitrate, 0.25 IU/mL isocitrate dehydrogenase, 1 mM NADP, and 3.0 mg/mL of kidney microsomal protein. Incubations were performed at 37°C for a 20-min period (a range where the reaction was linear with time). Results are expressed as means ± SD, and the statistical significance of differences between mean values was assessed by analysis of variance (ANOVA) coupled with Student-Newman-Keuls test at the 5% level of significance.

RESULTS

Table 1 shows the different doses of fish oil provided to the rats daily for seven days, using 0.5 g/kg body weight initially and then increasing to 7.5 g/kg body weight. The body and kidney weight were not significantly altered by fish oil (220 ± 10 and 1.09 ± 0.10 g, respectively). The liver weight, however, increased about 20% in all the groups treated. The kidney microsomal protein was not significantly altered by fish oil (7.5 ± 0.81 mg/g kidney); in contrast, an increase of about 27% in liver microsomal protein was observed with all the doses used (Table 1). It is known that the kidney content of cytochrome P-450 is lower than that of the liver (6,7). In the control rats, the cytochrome P-450 concentration in kidney (0.98 ± 0.24 nmol/g wet weight) was one-fifth that found in liver (5.28 ± 0.60). Although no effect on the renal cytochrome P-450 concentration was detectable, all four fish oil doses increased the hepatic concentration of cytochrome P-450 by 27% (Table 1). Because the dietary fish oil also increased the liver size by 22%, all four doses of fish oil increased the total liver microsome P-450 content by about 55% (i.e., from 62.3 ± 18.1 nmol cytochrome P-450/liver to 101.95 ± 20.4 control and dose I values, respectively). Surprisingly, the effects on liver weight, the cytochrome P-450 concentration, and content did not increase with the fish oil dosage. There were no significant differences in the liver weight, microsomal protein, and cytochrome P-450 content between the fish oil doses I–IV, as assessed by ANOVA coupled with the Student-Newman-Keuls test.

TABLE 1
Effect of Different Doses of Fish Oil on the Liver Weight, Microsomal Protein, and Cytochrome P-450 Content

Group	Fish oil (g/kg)[a]	Liver weight (g)[b]	Microsomal protein (mg/g liver)[c]	Cytochrome P-450 (nmol/g liver)[c]
Control	0.00	11.8 ± 1.10	9.1 ± 1.02	5.28 ± 0.6
Dose I	0.50	14.4 ± 1.50[d]	11.8 ± 1.10[d]	7.08 ± 0.8[d]
Dose II	1.00	14.0 ± 1.60[d]	11.6 ± 0.90[d]	6.38 ± 0.6
Dose III	5.00	14.2 ± 0.50[d]	11.9 ± 1.30[d]	7.14 ± 1.1[d]
Dose IV	7.50	14.8 ± 1.50[d]	11.0 ± 1.40[d]	6.27 ± 0.6

[a]Fish oil containing 20% eicosapentaenoic acid and 10% docosahexaenoic acid was provided daily in the doses indicated during seven days, and are expressed as g oil/kg body weight.
[b]Values are the means ± SD of at least 20 rats.
[c]Values are the means ± SD of at least five different experiments.
[d]Significantly different from control ($P < 0.05$) as assessed by analysis of variance coupled with Student-Newman-Keuls test.

The effect of fish oil on liver microsomal metabolism of lauric acid is shown in Table 2. Fish oil had no effects on renal microsome capacity to ω- and ωl-hydroxylate lauric acid, which remained at 0.38 ± 0.08 and 0.72 ± 0.10 nmol product/min/mg protein, respectively. In liver, however, a significant increase of about 43% was seen in the overall rate of

TABLE 2
Effect of Fish Oil on the Lauric Acid Hydroxylation Catalyzed by Rat Liver Microsomes[a]

	Metabolites (nmol product/min/mg protein)		
	11-Hydroxy lauric acid	12-Hydroxy lauric acid	Total
Control	0.50 ± 0.13	0.95 ± 0.16	1.44 ± 0.25
Dose I	0.52 ± 0.12	1.11 ± 0.25	1.63 ± 0.40
Dose II	0.44 ± 0.10	0.87 ± 0.12	1.31 ± 0.18
Dose III	0.82 ± 0.05	1.25 ± 0.10	2.07 ± 0.28[b]
Dose IV	0.67 ± 0.12	1.38 ± 0.15	2.05 ± 0.29[b]

[a]Values are the means ± SD of at least five different experiments.
[b]Significantly different from control ($P < 0.05$) as assessed by analysis of variance coupled with Student-Newman-Keuls test.

TABLE 3
Effect of Fish Oil on the Aminopyrine *N*-Demethylation Catalyzed by Rat Kidney and Liver Microsomes[a]

Group	(nmol product/min/mg protein)	
	Kidney	Liver
Control	0.06 ± 0.01	1.53 ± 0.21
Dose III	0.17 ± 0.02[b]	2.96 ± 0.13[b]
Dose IV	0.23 ± 0.05[b]	3.34 ± 0.36[b]

[a]Values are the means ± SD of at least five different experiments.
[b]Significantly different from control ($P < 0.05$) as assessed by analysis of variance coupled with Student-Newman-Keuls test.

lauric acid hydroxylation with the highest fish oil doses used (5 and 7.5 g/kg body weight). Only the effect of fish oil dose II was significantly different from doses III and IV. Because the fish oil also increased the amount of microsomal protein by an average of 27% (Table 1), the total hydroxylase activity in liver increased from a control value of 13.10 ± 1.44 nmol product/min/g liver to 19.23 ± 2.12, 15.20 ± 1.50, 24.63 ± 5.21, and 22.55 ± 5.24, with doses I, II, III, and IV, respectively. Thus, fish oils increased both the total cytochrome content and the total lauric acid hydroxylase activity in liver. In all the groups studied, the metabolite produced in a major proportion was the 12-hydroxy lauric acid (ω-derivative).

Aminopyrine *N*-demethylation was assayed in order to study the effect of fish oil on the activity of a different cytochrome P-450 family than CYP4A. Only fish oil doses III and IV were used in these studies. The renal metabolism of aminopyrine was lower than that of the hepatic tissue, the control group being about 4% of the liver values (Table 3). The metabolism of this exogenous substrate was significantly increased in both organs in rats treated with the higher doses of fish oil. The aminopyrine metabolism increased by about 3–4-fold in kidney and about 2-fold in liver in respect to the controls. There were significant differences between the effect of fish oil doses III and IV in both organs.

The liver and kidney microsomal turnover rates for aminopyrine and lauric acid was compared. In all the groups studied, the renal turnover rate of aminopyrine was lower than the hepatic one, being about 17% of the liver values in the control group (0.46 ± 0.07 and 2.65 ± 0.08 nmol product/min/nmol cytochrome P-450 for the kidney and liver values, respectively). In contrast, the kidney exhibited a higher metabolism of lauric acid. The turnover rate (nmol lauric acid product/min/nmol cytochrome P-450) in kidney (8.46 ± 2.01) was about threefold that in liver (2.48 ± 0.40).

DISCUSSION

In our studies, the liver and kidney respond in a different way to fish oil ingestion. Whereas the aminopyrine metabolism in both organs was increased by fish oil, the lauric acid metabolism increased only in the liver microsomes. Little is known about the fish oil effect on the kidney microsomal activity. In contrast to liver, kidney cytochrome P-450 is poorly induced by phenobarbital and clofibrate (16,17). Also, the response to peroxisome proliferators, such as di(ethylhexyl)pthalate, is different in liver and in kidney (18).

In relation to the effect of fish oil on the liver microsomal lauric acid metabolism, our results are similar to those obtained by Nilsson *et al.* (19). By feeding rats a diet with 20% fish oil (w/w) for one week, they obtained a more acute effect in increasing the liver lauric acid metabolism (about 2.5-fold compared to controls). The difference between our results and those reported by Nilsson *et al.* (19) might be attributed to the different fish oil origin or the doses ingested. Mounie *et al.* (20), by feeding rats a diet with a 6% fish oil content, reported an increased cytochrome P-450 content and bilirubin glucuronosyl transferase activity in liver.

Part of the clinical properties attributed to EPA (20:5n-3) are due to its structural similarity to arachidonic acid (20:4n-6) (21,22). Cytochrome P-450 participates in the metabolism of arachidonic acid, generating ω-derivates, epoxides, and others metabolites with biological activity (2,23). Several cytochrome P-450 isoenzymes involved in the metabolism of arachidonic acid have been purified in the rat liver and kidney (24). With the increasing interest in the health benefits of marine oils containing n-3 polyunsaturated fatty acids, it has been shown that EPA and DHA also can be metabolized by cytochrome P-450 *in vitro* (25). Urinary excretion of epoxides and diols derived from EPA during the fish oil ingestion by humans has been reported (26). In the microsomal metabolism of EPA, the participation of CYP4A family, specifically to metabolize fatty acids in the ω-position, is probable.

It is known that the levels and activities of cytochrome P-450 enzymes are affected by the diet (1,2). Our studies show that the liver and kidney respond in a different way to a dietary factor such as fish oil. The fish oil increased the lauric acid metabolism only in the liver. The aminopyrine metabolism, however, was induced in both organs. These results suggest that fish oil increases the activity of two different liver cytochrome P-450 families. The activity of kidney lauric acid 11- and 12-hydroxylase, however, was not modulated by this factor. More experimental work is needed to explore the biological significance of these selective changes.

ACKNOWLEDGMENTS

We thank Dr. Eugenia Del Villar and Catherine Allende for their assistance in the preparation of the manuscript. This work was supported by Grant B-3178 from Departamento Tecnico de Investigacion from the Universidad de Chile and Grant 1950-699 from Fondecyt.

REFERENCES

1. Gil, L., Orellana, M., Vasquez, H., and Silva, M. (1988) Nutrition-Related Alterations in Liver Microsomal Testosterone Hydroxylases, *Intern. J. Andrology 11*, 339–348.
2. Orellana, M., Valdes, E., Capdevila, J., and Gil, L. (1989) Nutritionally Triggered Alterations in the Regiospecificty of Arachidonic Acid Oxygenation by Rat Liver Microsomal Cytochrome P-450, *Arch. Biochem. Biophys. 274*, 251–258.

3. Hawkins, J.M., Jones, W.E., Bonner, F.W., and Gibson, G.G. (1987) The Effect of Peroxisome Proliferators on Microsomal, Peroxisomal, and Mitochondrial Enzyme Activities in the Liver and Kidney, *Drug Metab. Rev. 18*, 441–551.
4. Orellana, M., Fuentes, O., Rosenbluth, H., and Valdes, E. (1992) Modulation of Rat Liver Peroxisomal and Microsomal Fatty Acid Oxidation by Starvation, *FEBS Lett. 310*, 193–196.
5. Orellana, M., Fuentes, O., and Valdes, E. (1993) Starvation Effect on Rat Kidney Peroxisomal and Microsomal Fatty Acid Oxidation. A Comparative Study Between Liver and Kidney, *FEBS Lett. 322*, 61–64.
6. Ellin, A., Jakobsson, S.V., Schenkman, J.B., and Orrenius, S. (1972) Cytochrome P-450 of Rat Kidney Cortex Microsomes: Its Involvement in Fatty Acid ω and (ω-1) Hydroxylation, *Arch. Biochem. Biophys. 150*, 64–71.
7. Jakobsson, S., Thor, H., and Orrenius, S. (1970) Fatty Acid Inducible Cytochrome P-454 of Rat Kidney Cortex Microsomes, *Biochem. Biophys. Res. Com. 39*, 1073–1080.
8. Rustan, A.C., Christiansen, E.N., and Drevon, C.A. (1992) Serum Lipids, Hepatic Glycerolipid Metabolism and Peroxisomal Fatty Acid Oxidation in Rats Fed Omega-3 and Omega-6 Fatty Acids, *Biochem. J. 283*, 333–339.
9. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M., and Berge, R.K. (1993) Docosahexaenoic Acid Shows No Triglyceride-Lowering Effects But Increases the Peroxisomal Fatty Acid Oxidation in Liver of Rats, *J. Lipid Res. 34*, 13–22.
10. Nilsson, A., Prydz, K., Rortveit, T., and Christiansen, E.N. (1987) Studies on the Interrelated Stimulation of Microsomal Omega-Oxidation and Peroxisomal Beta-Oxidation in Rat Liver with a Partially Hydrogenated Fish Oil Diet, *Biochem. Biophys. Acta. 920*, 114–119.
11. Thomassen, M.S., Christiansen, E.N., and Norum, K.R. (1982) Characterization of the Stimulatory Effect of High-Fat Diets on Peroxisomal Beta-Oxidation in Rat Liver, *Biochem. J. 206*, 195–202.
12. Gil, L., Orellana, M., Mancilla, J., and Vasquez, H. (1986) Use of a New HPLC Method in Rat Liver Microsomal Testosterone Monooxygenation and Its Application to Study the Sex Dependent Expression of Several Hydroxylases, *Anal. Lett. 19*, 2261–2275.
13. Omura, T., and Sato, R. (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes, *J. Biol. Chem. 239*, 2379–2385.
14. Jacobsson, S.V., and Cinti, D.L. (1973) Studies on the Cytochrome P-450-Containing Mono-Oxygenase System in Human Kidney Cortex Microsomes, *J. Pharmacol. Exp. Ther. 185*, 226–230.
15. Nash, T. (1953) The Colorimetric Estimation of Formaldehyde by Means of the Hantzsch Reaction, *Biochem. J. 55*, 416–421.
16. Sharma, K., Lake, B.G., Makowsky, R., Bradshaw, T., Earnshaw, D., Dale, J.W., and Gibson, G.G. (1989) Differential Induction of Peroxisomal and Microsomal Fatty Acid-Oxidizing Enzymes by Peroxisome Proliferators in Rat Liver and Kidney, *European J. Biochem. 184*, 69–78.
17. Okey, A.B. (1990) Enzyme Induction in the Cytochrome P-450 System, *Pharmacol. Ther. 45*, 241–298.
18. Reubsaet, F.A., Veerkamp, J.H., Bruckwilder, M.L., Trijbels, J.M., and Monnens, L.A. (1991) Peroxisomal Oxidases and Catalase in Liver and Kidney Homogenates of Normal and Di(ethylhexyl)phthalate-Fed Rats, *Int. J. Biochem. 23*, 961–967.
19. Nilsson, A., Arey, H., Pedersen, J.I., and Christiansen, E.N. (1986) The Effect of High-Fat Diets on Microsomal Lauric Acid Hydroxylation in Rat Liver, *Biochem. Biophys. Acta 879*, 209–214.
20. Mounie, J., Goudonnet, H., Prevost, C., Champion, L., Truchot, R.C., and Escousse, A. (1990) Nutritional Factors of Microsomal Enzymatic Induction: Influence of Lipidic Components of the Diet, *Eur. J. Drug Metab. Pharmacokinet. 15*, 265–271.
21. Chee, K.M., Gong, J.X., Good Rees, D.M., Meydani, M., Ausman, L., Johnson, J., Siguel, E.N., and Shaefer, E.J. (1990) Fatty Acid Content of Marine Oil Capsules, *Lipids. 25*, 523–528.
22. Croft, K.D., Codde, J.P., Barden, A., Vandongen, R., and Beilin, L.J. (1985) Onset of Changes in Phospholipid Fatty Acid Composition and Prostaglandin Synthesis Following Dietary Manipulation with n-6 and n-3 Fatty Acids in the Rat, *Biochem. Biophys. Acta 834*, 316–323.
23. Capdevila, J.H., Falk, J.R., and Estabrook, R.W. (1992) Cytochrome P450 and the Arachidonate Cascade, *FASEB J. 6*, 731–736.
24. Imaoka, S., Tanaka, S., and Funae, Y. (1989) Omega- and (omega-1)-Hydroxylation of Lauric Acid and Arachidonic Acid by Rat Renal Cytochrome P-450, *Biochem. Int. 18*, 731–740.
25. VanRollins, M. (1990) Synthesis and Characterization of Cytochrome P-450 Epoxygenase Metabolites of Eicosapentaenoic Acid, *Lipids 25*, 481–490.
26. Knapp, H.R., Miller, A.J., and Lawson, J.A. (1991) Urinary Excretion of Diols Derived from Eicosapentaenoic Acid During n-3 Fatty Acid Ingestion by Man, *Prostaglandins 42*, 47–54.

[Received December 29, 1994; Revision accepted August 31, 1995]

Effects of Peroxidative Stress and Age on the Concentration of a Deoxyguanosine-Malondialdehyde Adduct in Rat DNA

Harold H. Draper[a,*], Sanjiv Agarwal[a,1], Dagmar E. Voparil Nelson[a], Jae Joon Wee[a,2], A.K. Ghoshal[b] and Emmanual Farber[b]

[a]Department of Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1 and [b]Department of Pathology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

ABSTRACT: The effect of age and peroxidative stress on the concentration of a deoxyguanosine malondialdehyde adduct (dG-MDA) in rat tissues was investigated. Vitamin E deficiency had no effect on the dG-MDA content of liver DNA in rats fed a diet containing 10% corn oil. When 2% cod liver oil was added to this diet, the dG-MDA content of liver DNA doubled in the positive controls fed a high level of vitamin E (100 ppm dl-α-tocopherol), and there was a further increase when vitamin E was deleted. Neither iron nitrilotriacetate administration nor choline deficiency had any effect on the dG-MDA content of liver DNA. Carbon tetrachloride had a lowering effect. The failure of iron or carbon tetrachloride administration and of vitamin E deficiency to increase liver dG-MDA is consistent with their failure in previous experiments to affect the urinary excretion of dG-MDA. In contrast, these forms of peroxidative stress produce large increments in the urinary excretion of MDA adducts with lysine, reflecting increased formation and degradation of MDA-modified proteins. DNA appears to be protected from modification by MDA produced at extranuclear sites. The frequency of dG-MDA in different tissues of 4-month-old rats varied markedly: brain >> liver > kidneys and testes. Higher concentrations of dG-MDA were found in the liver and kidneys, but not the testes, of 25-month-old rats. The determinants of the concentration of dG-MDA in DNA merit further investigation.
Lipids 30, 959–961 (1995).

In previous studies on the products of malondialdehyde (MDA) reactions *in vivo*, two Schiff's base adducts with lysine, one acetylated and the other unacetylated, were found to be the main forms of MDA excreted in human urine and in the urine of rats fed an MDA-free diet (1). These adducts reflect a reaction of MDA with the lysine residues of tissue and dietary proteins. Among several minor metabolites, a deoxyguanosine adduct with MDA (dG-MDA) was identified in urine (2) and in hydrolysates of rat liver DNA (3). Vitamin E deficiency, intraperitoneal administration of iron nitrilotriacetate (FeNTA), and oral administration of carbon tetrachloride resulted in marked increases in excretion of the lysine derivatives, signifying enhanced peroxidative modification of tissue proteins (1). In contrast, no effect was found on the excretion of dG-MDA (2).

In the present study, the effect of these treatments on the concentration of dG-MDA in rat liver DNA was investigated. In addition, the effect of choline deficiency, which gives rise to fluorescent pigments in the lipids of the nuclear membrane (4), was determined, as well as the dG-MDA content of DNA isolated from several tissues of young and old adult rats.

METHODS

Animal experiments: *Experiment 1.* Weanling rats were fed a vitamin E-deficient diet containing 10% molecular-distilled corn oil (1) with and without the addition of a high level of vitamin E (100 ppm dl-α-tocopherol). After six weeks, vitamin E depletion was confirmed using an autohemolysis test (81 vs. 17% in controls). The animals were euthanized and each liver was removed and frozen for dG-MDA analysis.

Experiment 2. This experiment was similar to Experiment 1, except that 2% cod liver oil, a source of highly oxidizable n-3 polyunsaturated fatty acids (PUFA), was added to the 10% corn oil diet to increase the peroxidative stress imposed on the animals.

Experiment 3. Two groups of five rats (weighing ~100 g) that had been fed a stock diet from weaning age were treated with FeNTA (9 mg Fe/kg) or an equivalent amount of NTA administered intraperitoneally. Another group was given 10 μL of CCl_4 by stomach intubation. The animals were sacrificed 48 h later.

Experiment 4. Two groups of six F-344 male rats (weighing ~150 g) were fed a choline-deficient diet with and without the addition of 0.8% choline (4). They were euthanized three days later, at which time a peak in peroxidation of the nuclear membrane lipids of choline-deficient animals has been documented using chemiluminescence and spin-trap measurements (5). Prolonged feeding of this diet produces a

*To whom correspondence should be addressed.

[1]Present address: Department of Biological Sciences, Southern Methodist University, Dallas, TX 75275-0376.

[2]Present address: Korea Ginseng and Tobacco Research Institute, Taejeon, 305-345, Korea.

Abbreviations: FeNTA, iron nitrilotriacetate; MDA, malondialdehyde; dG-MDA, deoxyguanosine-malondialdehyde adduct; PUFA, polyunsaturated fatty acids.

Copyright © 1995 by AOCS Press

100% incidence of putative preneoplastic hepatocyte nodules and a 50% incidence of hepatocellular carcinoma (4).

Experiment 5. The liver, kidneys, and testes from rats 4 vs. 25 months of age were analyzed to determine the relationship between aging and the dG-MDA content of DNA. The brain also was taken for analysis.

dG-MDA analysis. The procedures used for the isolation and hydrolysis of DNA and the determination of dG-MDA have been described previously (3). One-gram samples were used except in the case of brain, for which only 0.5-g samples were available. With the exception of the brain samples from the aged rats, clean fluorescence peaks were obtained for dG-MDA in the high-performance liquid chromatography chromatograms.

RESULTS AND DISCUSSION

The results of Experiments 1, 2, 3, and 4 are summarized in Table 1. Vitamin E depletion on the 10% distilled corn oil diet had no significant effect on the dG-MDA content of liver DNA. The excretion of total MDA, estimated by high-performance liquid chromatography as its thiobarbituric acid derivative, was higher in the vitamin E-deficient group (13.9 vs. 8.1 μg/24 h) ($P < 0.05$). This finding is in agreement with previous results (6). Adding 2% cod liver oil to the corn oil diet was associated with an increase in the dG-MDA content of hepatic DNA ($P = 0.02$). Neither FeNTA nor choline deficiency significantly affected dG-MDA content, and the hepatotoxic agent CCl_4 had a depressing effect.

The dG-MDA content of liver and kidney DNA, but not of testicular DNA, was higher in the 25-month-old rats than in the 4-month-old rats (Table 2). By far the highest concentration of dG-MDA was found in the brain. The yield of DNA from the brains of the old adults was too small and the analytical values too variable to provide a reliable estimate of their dG-MDA content.

TABLE 1
Effect of Vitamin E Depletion and the Administration of Lipid Oxidants on the Deoxyguanosine-Malondialdehyde Adduct (dG-MDA) Content of Rat Liver DNA

	dG-MDA[a]	*P*
Experiment 1 (corn oil diet)		
+E	4.5 ± 1.5	—
−E	5.1 ± 1.0	>.05
Experiment 2 (corn plus cod liver oil diet)		
+E	10.9 ± 1.5	—
−E	17.9 ± 2.0	.02
Experiment 3 (stock diet)		
Control	6.6 ± 0.8	—
FeNTA	6.8 ± 0.6	>.05
CCl_4	3.6 ± 0.9	<.05
Experiment 4 (choline-deficient diet)		
+Choline	7.7 ± 1.0	—
−Choline	8.9 ± 1.3	>.05

[a]pmol/100 μg DNA. Mean ± SEM, n = 5.

TABLE 2
The dG-MDA Content of DNA in Tissues of Young Adult and Aged Rats (pmol/100 μg DNA)[a]

	Age		
	4 mon	25 mon	*P*
Liver	9.9 ± 3.8	25.5 ± 6.5	<.05
Kidneys	1.7 ± 0.6	5.1 ± 1.2	<.05
Testes	1.7 ± 0.2	1.1 ± 0.4	>.05
Brain	62.0 ± 8.6	—[b]	—

[a]Mean ± SEM, n = 6. Abbreviation as in Table 1.
[b]Not determined.

The findings indicate that liver DNA is protected from modification by the increased amounts of MDA generated in vitamin E deficiency and by the administration of chemical catalysts of hepatic lipid peroxidation. Judging from the large increases in excretion of MDA adducts with lysine under the same conditions (1), DNA differs in this respect from tissue proteins. Both purified proteins and DNA react readily with MDA *in vitro*, but neither liver nor urine analysis has yielded consistent evidence of increased dG-MDA generation *in vivo* under the conditions of peroxidative stress imposed by these treatments. It is noteworthy that the increased fluorescence of nuclear membrane lipids caused by feeding a choline-deficient diet is not prevented by vitamin E or synthetic antioxidants, whereas it is completely prevented by choline and the free radical trapping agents *tert*-nitrosobutane and α-phenyl-*tert*-butylnitrone (5,7).

PUFA and vitamin E are concentrated in the mitochondria and endoplasmic reticulum of hepatocytes which, presumably, are therefore the main sites of MDA generation. These organelles contain aldehyde oxidases with a high affinity for MDA. Most of the MDA formed in hepatocytes appears to be either oxidized to CO_2 *in situ* or to react with proteins in the cytosol and extracellular fluids. These soluble proteins may constitute a defense barrier against modification of nuclear DNA by MDA and other aldehydes. Serum albumin reacts avidly with MDA *in vitro*, and most of the MDA in plasma is appears to be bound to albumin (8).

Although binding of MDA by soluble proteins may explain the prevention of DNA damage by MDA generated at extranuclear sites, it does not explain the apparent lack of increased MDA generation in the nucleus in vitamin E deficiency. The effect of vitamin E deficiency on its concentration in purified nuclei does not appear to have been investigated. It is also possible that the nuclear membrane is impermeable to the oxidants employed in this study.

If nuclear lipids are not vulnerable to lipid oxidants or a deficiency of antioxidants, some other explanation is necessary to explain the differences in the dG-MDA content of DNA among tissues and at different ages. No dG-MDA could be found in a commercial sample of calf thymus DNA (Sigma, St. Louis, MO) (data not shown). The increased values obtained for the dG-MDA content of liver DNA isolated from rats fed 2% cod liver oil suggests an effect of the concentration of highly oxidizable fatty acids of the n-3 series. In

a previous study (9), addition of cod liver oil to a corn oil-based diet produced an increase in fasting MDA excretion, whereas increments in corn oil in the diet had no effect. An effect of highly unsaturated fatty acids of the n-3 series on the dG-MDA content of brain DNA is suggested by the high concentrations of these acids and iron in the brain (9). The effect of the intake of highly unsaturated PUFA on the fatty acid composition and vitamin E content of nuclear membrane lipids, as well as on the binding of MDA, a confirmed mutagen in bacterial and mammalian cells (10), to DNA *in vivo* is worthy of further investigation.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada, the National Cancer Institute of Canada, and the Medical Research Council of Canada.. The authors are grateful for a gift of synthetic dG-MDA from Hiroshi Seto (Tokyo, Japan). Tissues from young and old adult rats were generously provided by George Roth, U.S. National Institute on Aging (Baltimore, MD).

REFERENCES

1. Mahmoodi, H., Hadley, M., Chang, Y-X., and Draper, H.H. (1995) Increased Formation and Degradation of Malondialdehyde-Modified Proteins Under Conditions of Peroxidative Stress, *Lipids 30*, 963–966.
2. Agarwal, S., Wee, J.J., Hadley, M., and Draper, H.H. (1994) Identification of a Deoxyguanosine-Malondialdehyde Adduct in Rat and Human Urine, *Lipids 29*, 429–432.
3. Agarwal, S., and Draper, H.H. (1992) Isolation of a Deoxyguanosine-Malondialdehyde Adduct from Rat Liver DNA, *Free Radical Biol. Med. 13*, 695–699.
4. Ghoshal, A.K., and Farber, E. (1984) The Induction of Liver Cancer by Dietary Deficiency of Choline and Methionine Without Added Carcinogens, *Carcinogenesis 5*, 1367–1370.
5. Ghoshal, A.K., Rushmore, T.H., Buc-Calderon, P., Roberfroid, M., and Farber, E. (1990) Prevention by Free Radical Scavenger AD_5 of Prooxidant Effects of Choline Deficiency, *Free Radical Biol. Med. 8*, 3–7.
6. Danakoti, S.N., and Draper, H.H. (1987) Response of Urinary Malondialdehyde to Factors that Stimulate Lipid Peroxidation *in vivo*, *Lipids 22*, 643–646.
7. Rushmore, T.H., Ghazarian, D.M., Subrahmanyan, V., Farber, E., and Ghoshal, A.K. (1987) Probable Free Radical Effects on Rat Liver Nuclei During Early Hapatocarcinogenesis with a Choline-Devoid Low Methionine Diet, *Cancer Res. 47*, 6731–6740.
8. Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., and Hadley, M. (1993) A Comparative Evaluation of Thiobarbituric Acid Methods for the Determination of Malondialdehyde in Biological Materials, *Free Radical Biol. Med. 15*, 353–363.
9. Floyd, R.A., Zaleska, M.M., and Harmon, H.J.(1984) Possible Involvement of Iron and Oxygen Free Radicals in Aspects of Aging in the Brain, in *Free Radicals in Molecular Biology, Aging, and Disease* (Armstrong, D., Sohal, R.S., Cutler, R.G., and Slater, T.F., eds.) pp. 143–161, Raven Press, New York.
10. Basu, A.K., and Marnett, L.J. (1983) Unequivocal Demonstration That Malondialdehyde is a Mutagen, *Carcinogenesis 4*, 331–333.

[Received August 6, 1994, and in final revised form May 5, 1995; Revision accepted July 3, 1995]

Increased Formation and Degradation of Malondialdehyde-Modified Proteins Under Conditions of Peroxidative Stress

Haleh Mahmoodi, Mary Hadley[1], Yuan-Xun Chang[2] and Harold H. Draper*

Department of Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1, Canada

ABSTRACT: The effect of increased *in vivo* lipid peroxidation on excretion of the main urinary metabolites of malondialdehyde (MDA) was investigated. Peroxidative stress in the form of vitamin E deficiency or the administration of iron nitrilotriacetate or carbon tetrachloride was imposed on rats fed an MDA-free diet. Significant increases were observed in excretion of the lysine–MDA adduct ε-propenal lysine, its *N*-acetyl ester, and free MDA. Under the conditions imposed, the increments in excretion of the lysine adducts reflect increased peroxidative modification of tissue proteins *in vivo*. These adducts also were found to be the main forms of MDA excreted in human urine. Reacting ^{14}C-bovine serum albumin (BSA) with MDA resulted in its accelerated proteolysis *in vitro* by soluble enzyme preparations derived from human erythrocytes and rat liver mitochondria. The increments observed were similar to those reported for the hydrolysis of BSA following its exposure to hydroxyl radicals. The results show that lipid peroxidation *in vivo* results in peroxidative damage to tissue proteins and indicate that such proteins are subject to an accelerated rate of proteolysis.
Lipids 30, 963–966 (1995).

In studies on the reactions of malondialdehyde (MDA) *in vivo,* two Schiff's base adducts with lysine, one acetylated (1) and the other unacetylated (2), were found to comprise about 75% of the total malondialdehyde (MDA) excreted in the urine of rats fed an MDA-free diet. These compounds also constitute the main forms of MDA excreted in human urine. On normal diets, the amounts formed endogenously are augmented by release of the lysine adduct ε-propenal lysine (ε-PL) from food proteins in the course of digestion and its partial acetylation to form *N*-α-acetyl propenal lysine (APL) in the tissues (3).

In Part I of the present study, the effect of peroxidative stress on excretion of the two lysine adducts and free MDA was investigated in rats. In Part II the effect of reacting serum albumin with MDA on its rate of hydrolysis *in vitro* by a proteolytic enzyme from human red blood cells (RBC) and rat liver mitochondria was determined.

EXPERIMENTAL PROCEDURES

Part I. Two groups of six weanling rats of the Charles River strain were fed a vitamin E-deficient diet containing 10% molecular-distilled corn oil (4) for 10 wk. Vitamin E depletion was confirmed by an autohemolysis test (4). Controls received a supplement of 100 ppm dl-α-tocopherol. Urine was collected daily during the final week and frozen at –20°C.

In a second experiment, a group of six growing rats weighing ~150 g that had been fed a stock diet from weaning age were injected intraperitoneally with 9 mg Fe/kg as iron nitrilotriacetate (4). Controls were injected with an equivalent amount of nitrilotriacetate. Another group was treated with 10 μL CCl_4 by gastric intubation. Urine was collected under toluene for the following two days and frozen at –20°C. To avoid interference in the urinalysis by ε-PL released from dietary proteins in the course of digestion (3), the animals were switched to the MDA-free high vitamin E diet used in the first experiment two days prior to and during the collection period.

Urinalysis. Fifty-mL aliquots of urine were fractionated on a 2.3 x 45 cm AG-1 anion exchange column as described previously (1). The column was developed using a 0–2 M NaCl gradient and 5-mL fractions were collected. Every fifth fraction was analyzed for MDA by a spectrophotometric thiobarbituric acid (TBA) procedure (5). This procedure yields MDA values for urine that are indistinguishable from those obtained using a high-performance liquid chromatography (HPLC) procedure (5). The urine samples from each rat were chromatographed individually and a composite elution profile of MDA compounds was constructed for each treatment. The significance of group differences in the excretion of ε-PL, APL, and MDA was determined by analysis of variance of the data for their respective elution peaks and for those of their controls.

For comparative purposes, 24-h urine samples were collected from five adult humans and 50-mL aliquots were chromatographed by the same procedure. To minimize the excretion of lysine–MDA adducts of dietary origin, the subjects consumed a protein-free diet for 24 h prior to and during the collection.

[1]Present address: Food and Nutrition Department, North Dakota State University, Fargo, ND 58105-5057.

[2]Present address: Department of Occupational Health, School of Public Health, Beijing Medical University, Beijing, China.

*To whom correspondence should be addressed.

Abbreviations: APL, *N*-α-acetyl propenal lysine; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; ε-PL, ε-propenal lysine; RBC, red blood cell; TBA, thiobarbituric acid, TCA, trichloracetic acid.

Copyright © 1995 by AOCS Press

Part II. Preparation of MDA-modified ^{14}C-bovine serum albumin (BSA). MDA was prepared fresh by acid hydrolysis of tetramethoxypropane (1N HCl for 40 min at 40°C); 0.5 mL of a 0.05 mM solution of MDA (1.9 μg) was incubated with 0.5 mL of a 7.6×10^4 mM solution of ^{14}C-labeled BSA (^{14}C-BSA, specific activity 30.1 μCi/mg, 16.6 μg; Sigma Chemical Co., St. Louis, MO) in 50 mM phosphate/citrate buffer, pH 4.3, at 37°C overnight. Unreacted MDA was removed by vacuum desiccation at 37°C for 16 h. Preliminary experiments showed that ≥99% of free MDA was removed under these conditions. The residue was taken up in buffer, and 10 μL aliquots were frozen at –70°C. MDA in the protein preparations was determined as the TBA derivative using an HPLC procedure that is specific for this compound (5). This procedure revealed that the unreacted ^{14}C-BSA contained 20 ppm MDA. The MDA-reacted ^{14}C-BSA used in the RBC and mitochondrial experiments contained 120 and 60 ppm MDA, respectively.

RBC assay. The enzyme was prepared from human RBC according to the procedure of Davies and Goldberg (6) for rabbit erythrocytes. The protein content was determined by the Lowry procedure and 60 μL of the enzyme preparation (~5 mg protein), 10 μL (0. 10 μg) of ^{14}C-BSA, and 80 μL of Hepes buffer, pH 7.8, were incubated at 37°C for up to 6 h. One volume of 20% trichloracetic acid (TCA) was added, and the mixture was kept on ice for 30 min. Intact proteins and large peptides were removed by centrifugation at 16,000 × *g* for 10 min. Radioactivity in the clear supernate was quantified by scintillation counting (Beckman LS 3810; Beckman Instruments, Mississauga, Ontario, Canada) using 10 mL of Ecolume cocktail (ICN Biochemicals, Inc., Mississauga, Ontario, Canada). The % recovery of radioactivity in the TCA-soluble fraction was calculated.

Rat liver mitochondria assay. The livers were homogenized using a Potter-Elvehjem Teflon homogenizer. Mitochondria were prepared according to Pedersen *et al.* (7) in a medium consisting of 0.07 M sucrose, 0.22 M D-mannitol, 0.002 M Hepes buffer, and 0.05% BSA. The enzyme was prepared and the assays carried out according to the procedures of Marcillat *et al.* (8). Sixty μL of the mitochondrial preparations (0.4 mg of protein), 10 μL of ^{14}C-BSA solution, and 60 μL of 50 mM Hepes buffer, pH 7.8, were incubated at 37°C for up to 6 h. Then 60 μL of a carrier solution of unlabelled BSA (4.5 mg) was added, followed by 190 μL of 20% TCA. The mixture was kept on ice for 30 min before centrifuging at 16,000 × *g* for 10 min. The recovery of radioactivity in the TCA-soluble fraction was used as a measure of proteolysis.

RESULTS

Part I. The elution profiles obtained for rat urine MDA and its conjugates are shown in Figure 1. They demonstrate that rats fed a vitamin E-deficient diet or dosed with Fe or CCl_4 excreted substantially larger amounts of ε-PL, APL, and free MDA than the controls. The enhanced excretion of free MDA reflects an increase in MDA production *in vivo* and the enhanced excretion of the lysine adducts a resulting increase in its reactions with tissue proteins. The elution profile for one 24-h sample of human urine is shown in Figure 2. It contains major peaks for APL and ε-PL and an unusually large peak for free MDA.

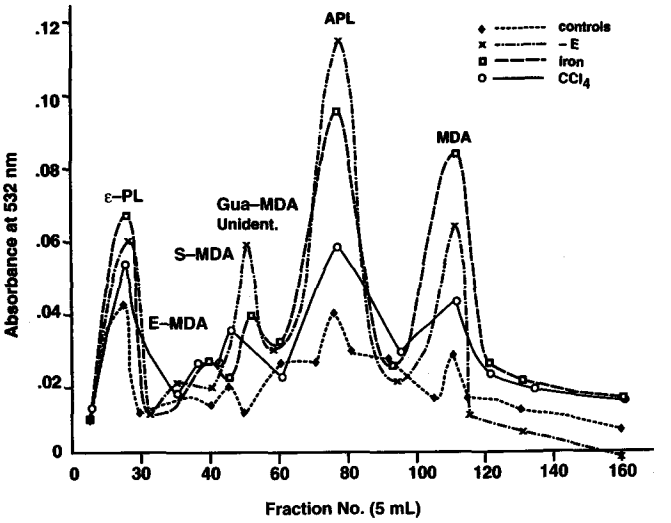


FIG. 1. Effect of vitamin E deficiency and iron or CCl_4 administration on the elution profile of malondialdehyde (MDA) metabolites obtained by anion exchange chromatography of rat urine. In addition to the lysine adducts and free MDA, the profile reflects the elution of minor amounts of adducts with ethanolamine (E–MDA), serine (S–MDA), guanine (Gua–MDA), and unidentified (Unident.) compounds. MDA was determined as the thiobarbituric derivative. In the interest of clarity, not all data points are shown. APL, *N*-α-acetyl propenal lysine; ε-PL, ε-propenal lysine.

Part II. RBC. There was a linear relationship between the amount of RBC protein used to hydrolyze ^{14}C-BSA (0–12.0

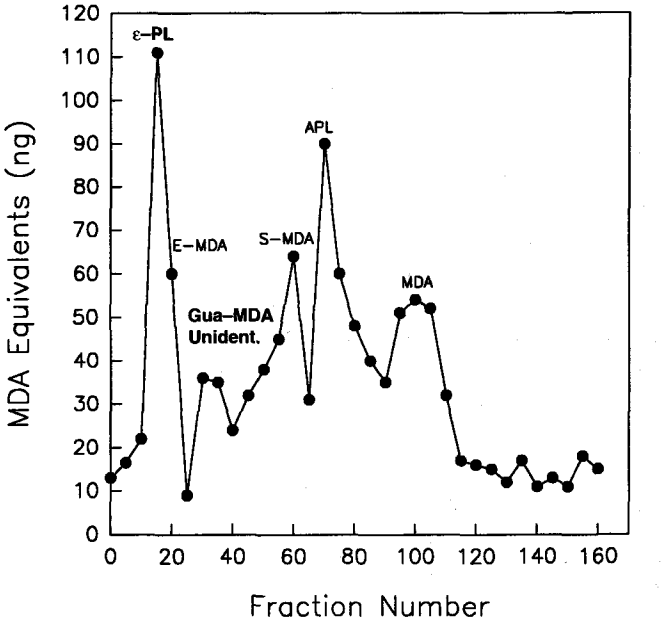


FIG. 2. Elution profile of MDA metabolites obtained by anion exchange chromatography of human urine. See Figure 1 for abbreviations.

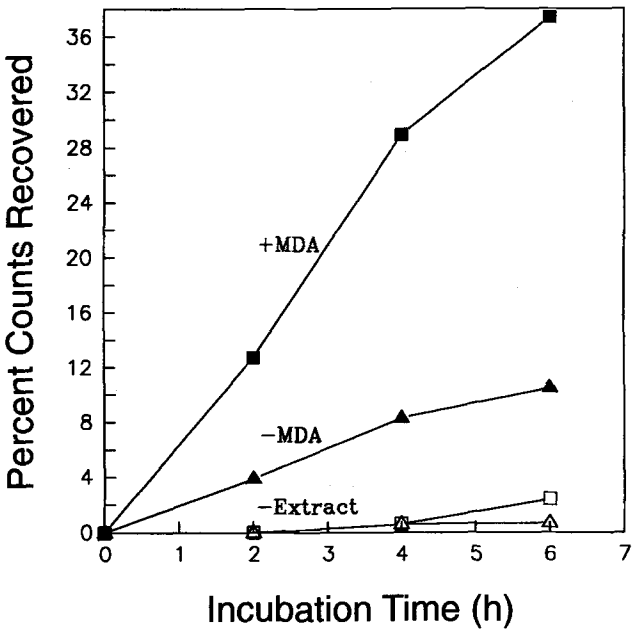


FIG. 3. Effect of reacting ^{14}C-bovine serum albumin with MDA on its rate of hydrolysis by the red blood cell enzyme. See Figure 1 for abbreviation.

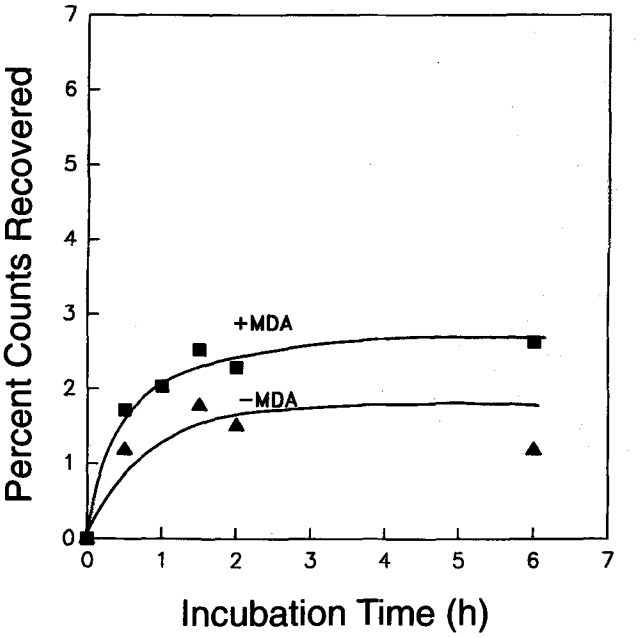


FIG. 4. Effect of reacting ^{14}C-bovine serum albumin with MDA on its rate of proteolysis by the mitochondrial enzyme. See Figure 1 for abbreviation.

mg) and the recovery of counts in the acid-soluble fraction. Five mg of protein was used for assay purposes. There was also a linear relationship between the amount of ^{14}C-BSA used (0–1. 0 μg) and the release of acid-soluble radioactivity (0–3,000 cdpm). The recovery of counts in the TCA fraction remained constant at 1.5% over the range of the assay.

The effect of reacting ^{14}C-BSA with MDA on its rate of hydrolysis by the RBC enzyme is illustrated in Figure 3. The release of counts from the reacted protein was threefold greater than that from the unreacted protein, a difference that was significant ($P < 0.0$ 1) using Student's *t*-test for paired values from five comparative assays. The release of counts in the enzyme blanks was nonsignificant ($P > 0.05$).

Liver mitochondrial assay. The recovery of acid-soluble counts increased linearly with increments in mitochondrial protein up to 0.2 mg, then reached a plateau. Assays were performed using 0.4 mg of protein. The yield of counts (0–300 cdpm) increased linearly with additions of up to 0.5 μg ^{14}C-BSA, and 0.10 μg was used in the assays. The fractional recovery of counts (1.2%) remained constant over the assay range (0–0.5 μg). The hydrolysis of MDA-modified ^{14}C-BSA proceeded at a rate approximately twice that of the unmodified protein (Fig. 4). This difference was found to be significant ($P < 0.01$) when the paired data from five comparative assays were analyzed. Degradation in the absence of the mitochondrial extract was negligible.

DISCUSSION

Normally, the main source of MDA in rat and human is the diet, from which it is absorbed primarily in the form of ε-PL (3). The excretion of free MDA and its lysine derivatives by rats fed a purified diet containing a nonperoxidizable fat (hydrogenated coconut oil) is only about 25% of that excreted on a stock diet of natural ingredients. However, the profile of MDA metabolites is similar, reflecting the predominant association of MDA with proteins in both the diet and the tissues. Neither the excretion of total MDA or of its lysine derivatives, therefore, is a useful indicator of *in vivo* lipid peroxidation, nor is the excretion of free MDA (9).

Free MDA (10), but not ε-PL (3), is mutagenic in bacterial and animal cells grown in culture. Administration of ε-PL to rats leads to marked increases in the excretion of ε-PL and APL, but not of free MDA, indicating that MDA is not a product of the metabolism of these compounds. MDA in food lipids therefore does not appear to pose a risk of mutagenicity in humans.

The reactivity of MDA with proteins has been known for many years. Crawford *et al.* (11) demonstrated in 1967 that MDA reacted with the lysine residues of *BSA in vitro.* The presence of 20 ppm MDA in a highly purified sample of ^{14}C-BSA may reflect a reaction with BSA *in vivo,* and the occurrence of ubiquitous reactions of MDA with tissue proteins is indicated by the predominance of lysine-MDA adducts in the urine of animals fed MDA-free diets (2). Most of the difference in values reported for the MDA content of animal tissues using TBA methods and methods for free MDA is attributable to hydrolysis of MDA from proteins during the TBA procedure (5). Serum albumin may have a physiological role as a scavenger for free MDA released into the circulation. No evidence was obtained in the present studies for the presence in urine of products of MDA reactions with amino acids other than lysine and the phospholipid base serine.

The clinical significance of the modification of tissue proteins by MDA, if any, is unknown. However, Haberland and co-workers (12) have shown that modification of 15% of the lysine residues of the apo-B100 moiety of plasma low-density lipoproteins by reactions with MDA impairs its recognition by the low-density lipoprotein receptor in human monocytes and macrophages and results in cholesterol deposition in the arterial walls. Reacting MDA with enzyme proteins *in vitro* has been reported by several investigators to result in inactivation, malfunction, or instability.

The responses of the RBC and liver enzymes to MDA-modified BSA observed in the present study are similar to those reported for their response to BSA modified by exposure to hydroxyl radical-generating systems (13). Although this similarity suggests that the protein damage caused by hydroxyl radicals may be mediated by initiation of lipid peroxidation, Davies and Goldberg (14) failed to find any evidence of increased lipid peroxidation in the course of their studies. The scavenger macroprotease may have a low specificity for the form of protein modification. Stadtman (15) demonstrated that oxidative damage to proteins in the presence of transition metal ions leads to the formation of carbonyl compounds and a concomitant susceptibility to degradation. Although MDA has been reported to cross-link proteins *in vitro* (16), there is no clear evidence that cross-linking occurs *in vivo* or that it results in protein cross-linking. Denaturing gel electrophoresis conducted on MDA-reacted BSA in the present study revealed no evidence of degradation. As postulated by Davies and Pacifici (17,18) to explain the accelerated proteolysis caused by hydroxyl radicals, MDA may alter the overall charge on proteins or expose previously hidden amino acid sequences that mark them for degradation.

ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. The helpful advice of Dr. Sanjiv Agarwal in the second part of these studies is appreciated.

REFERENCES

1. McGirr, L.G., Hadley, M., and Draper, H.H. (1985) Identification of N_{α} acetyl-N_{ε} (2-propenal) Lysine as a Urinary Metabolite of Malondialdehyde, *J. Biol. Chem. 260,* 15427–15431.
2. Draper, H.H., Hadley, M., Lissamore, L., Laing, N.M., and Cole, P.D. (1988) Identification of N_{ε} (2-propenal) Lysine as a Major Urinary Metabolite of Malondialdehyde, *Lipids 23,* 626–628.
3. Piche, L.A., Cole, P.D., Hadley, M., van den Bergh, R., and Draper, H.H. (1988) Identification of N_{ε} (2-propenal) Lysine as the Main Form of Malondialdehyde in Food Digesta, *Carcinogenesis 9,* 473–477.
4. Draper, H.H., Polensek, L., Hadley, M., and McGirr, L.G. (1984) Urinary Malondialdehyde as an Indicator of Lipid Peroxidation in the Diet and in the Tissues, *Lipids 19,* 836–843.
5. Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., and Hadley, M. (1993) A Comparative Evaluation of Thiobarbituric Acid Methods for the Determination of Malondialdehyde in Biological Materials, *Free Rad. Biol. Med. 15,* 353–363.
6. Davies, K.J.A., and Goldberg, A.L. (1987) Proteins Damaged by Oxygen Radicals are Rapidly Degraded in Extracts of Red Blood Cells, *J. Biol. Chem. 262,* 8227–8234.
7. Pedersen, P.L., Greenawalt, J.W., Reynafarje, B., Hullihen, J., Decker, G.L. Soper, J.W., and Bustamente, E. (1978) Preparation and Characterization of Mitochondria and Submitochondrial Particles of Rat Liver and Liver-Derived Tissues, in *Meth. Cell Biol.* (Prescott, D., ed.) Vol. 20, pp. 420–423, Academic Press, New York.
8. Marcillat, O., Zhang, Y., Lin S.W., and Davies K.J.A. (1988) Mitochondria Contain a Proteolytic System Which Can Recognize and Degrade Oxidatively-Denatured Proteins, *Biochem. J. 254,* 677–683.
9. Lee, H., Shoeman, D.W., and Csallany, A.S. (1992) Urinary Response to *in vivo* Lipid Peroxidation Induced by Vitamin E Deficiency, *Lipids 27,* 124-128.
10. Basu, A.K., and Marnett, L.J. (1983) Unequivocal Demonstration That Malondialdehyde Is a Mutagen, *Carcinogenesis 4,* 331–333.
11. Crawford, D.D., Yu, T.C., and Sinnhuber, R.O. (1967) Reaction of Malondialdehyde with Protein, *J. Food Sci. 32,* 332–335.
12. Haberland, M.E., Fogelman, A.M., and Edwards, P.A. (1982) Specificity of Receptor-Mediated Recognition of Malondialdehyde-Modified Low Density Lipoproteins, *Proc. Natl. Acad. Sci. USA 70,* 1712–1716.
13. Davies, K.J.A. (1988) Proteolytic Systems as Secondary Antioxidant Defenses, *Cellular Antioxidant Defence Mechanisms* (Chow, ed.) Vol. 2, pp. 25–67, CRC Press, Boca Raton.
14. Davies, K.J.A., and Goldberg, A.L. (1987) Oxygen Radicals Stimulate Intracellular Proteolysis and Lipid Peroxidation by Independent Mechanisms in Erythrocytes, *J. Biol. Chem. 262,* 8220–8226.
15. Stadtman, E.R. (1992) Protein Oxidation and Aging, *Science 257,* 1220–2008.
16. Shin, B.C., Huggins, J.W., and Carraway, K.L. (1972) Effects of pH, Concentration and Aging on the Malonaldehyde Reaction with Proteins, *Lipids 7,* 229–233.
17. Davies, K.J.A. (1987) Protein Damage and Degradation by Oxygen Radicals. I. General Aspects, *J. Biol. Chem. 262,* 9895–9901.
18. Pacifici, R.E., and Davies, K.J.A. (1991) Protein, Lipid and DNA Repair Systems in Oxidative Stress: the Free-Radical Theory of Aging Revisited, *Gerontology 37,* 166–180.

[Received November 7, 1994, and in final revised form August 14, 1995; Revision accepted August 23, 1995]

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) Temperature and Diet Effects on Trout, *J. Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) Temperature and Diet Effects on Trout, in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., Temperature and Diet Effects on Trout, *J. Biol. Chem.,* in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: All titles in References section should be capitalized. See examples above.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

AVAILABILITY OF PURIFIED OMEGA-3 FATTY ACIDS

Kilogram quantities of EPA and DHA ethyl ester capsules are available free of charge through the Fish Oil Test Materials Program, a joint effort of the National Oceanic and Atmospheric Administration and National Institutes of Health. These quality-assured and quality-controlled materials contain 94% EPA or 90% DHA. Ethyl esters of vegetable oils (olive, corn, and safflower) are available in capsule form as controls. Funded research projects studying the effect of

(continued on next page)

omega-3 fatty acids in health and disease, from all funding sources, are eligible to receive these capsules. For further information or to request a short application form, contact the Fish Oil Test Materials Program at 12501 Washington Ave., Rockville, MD 20852 (Phone: 301-443-9643; Fax: 301-594-0035).

CALENDAR

November 1995
XII International Symposium on Drugs Affecting Lipid Metabolism, Nov. 7–10, 1995, Westin Galleria and Westin Oaks Hotel, Houston, Texas, USA. Information: Giovanni Lorenzini Medical Foundation, 6550 Fannin, Suite 1423, Houston, TX 77030 (Phone: 713-797-0401; Fax: 713-796-8853).

February 1996
Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

July 1996
European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

July–August 1997
Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Low-Fat Diets Do Not Lower Plasma Cholesterol Levels in Healthy Men Compared to High-Fat Diets with Similar Fatty Acid Composition at Constant Caloric Intake

Gary J. Nelson*, Perla C. Schmidt and Darshan S. Kelley

Western Human Nutrition Research Center, USDA, ARS, Presidio of San Francisco, California 94129

ABSTRACT: In most studies reporting the effects of high-fat (HF) and low-fat (LF) diets on human plasma fatty acids (FA) and lipoprotein levels, the design involved adding to the diet an oil that had an FA composition (FAC) very different from the FAC of the control diet. Thus, it is difficult to determine if simply reducing the fat content of the diet without changing the dietary FAC changes the tissue FAC or alters plasma lipid levels. In this study, we fed diets that contained either 22 or 39% of calories from fat, but had no differences in their FAC, for 50 d to a group (n = 11) of healthy men (20–35 y). Thus, the polyunsaturated/saturated ratios (1.0) of the diets were identical as were the n-3/n-6 ratio and the monounsaturated-to-total fat ratios. The diets contained (wt% of total fat) approximately 28% saturated FA, 33% monounsaturated *cis*-FA, 6% monounsaturated *trans*-FA, 22% n-6 polyunsaturated FA, and 7% n-3 polyunsaturated FA, and 4% other minor FA. The diets consisted of natural foods and were formulated to contain 16 en% protein, either 45 or 62 en% carbohydrate (CHO) and at least the recommended daily allowance for all micronutrients. Both diets contained 360 mg of cholesterol per day. All subjects were given the HF diet for 20 d, and then six were placed on the LF and the other five remained on the HF diet for 50 d. The two groups were crossed-over for the remaining 50 d of the study. The subjects' baseline total cholesterol level was 173 mg/dl, after 50 d on the HF diet it was 177 mg/dl and after 50 d on the LF diet, 173 mg/dl. The differences were not significant, and there were no significant changes in either the LDL or HDL cholesterol levels with either diet. Triglyceride levels, and consequently very low density lipoprotein levels, rose significantly on the LF, higher CHO diet compared to the levels found in the subjects on the HF diet (91.5 and 66.4 mg/dl respectively, $P < 0.002$). The linoleic acid content of the plasma, platelets, and red blood cells was significantly ($P < 0.05$) reduced in the LF diet compared to HF diet, without any obvious physiological effects. Hence, many earlier observations indicating reductions in plasma lipid levels when people are on LF diets may be due to changes in the FAC of the diet, not the reduction in fat calories.

Lipids 30, 969–976 (1995).

*To whom correspondence should be addressed at USDA/ARS/WHNRC, P.O. Box 29997, Presidio of San Francisco, San Francisco, CA 94129.

Abbreviations: AA, arachidonic acid; CHO, carbohydrate; FA, fatty acid; FAC, fatty acid composition; GLC, gas–liquid chromatography; HDL, high density lipoproteins; HF, high-fat; LDL, low density lipoproteins; LF, low-fat; VLDL, very low density lipoprotein.

It has been reported for more than forty years that dietary saturated fatty acids (FA) will raise blood cholesterol levels, and conversely, dietary polyunsaturated FA will lower blood cholesterol levels (1–4). Keys *et al.* (5) and Hegsted *et al.* (6) almost simultaneously published formulas based on epidemiological data from various sources showing that mean cholesterol levels (hence, cardiovascular disease rates) in different populations could be directly related to the mean fat consumption of the population. Both Keys *et al.* (5) and Hegsted *et al.* (6) went on to point out that saturated fat raised blood cholesterol almost twice as much (on a per gram of intake basis) than polyunsaturated FA lowered it. Both formulae were similar. Only the constants and coefficients were different between the Keys *et al.* (5) and Hegsted *et al.* (6) equations. Another important finding was that dietary cholesterol had a minor effect on blood cholesterol levels, about 2 mg/dl per 100 mg of dietary cholesterol. Dietary monounsaturated FA (mainly oleic acid) were neutral; i.e., they didn't raise or lower blood FA levels. Also Keys *et al.* (5) and Hegsted *et al.* (6) suggested that the saturated eighteen carbon FA, stearic acid, didn't raise blood cholesterol significantly, a point largely ignored until a few years ago when Bonanome and Grundy (7) reinvestigated this issue and reached the same conclusion, although it should be noted that Dougherty *et al.* (8) recently published a report indicating that stearic acid is hypercholesterolemic if fed for longer periods.

Along with our knowledge of the specific cholesterolemic effects of dietary FA, high-fat (HF) diets, regardless of their specific FA composition, were considered hypercholesterolemic and consequently deleterious to cardiovascular health (9–11). This concept was based on two ideas: (i) that HF diets lead to obesity more easily than high carbohydrate (CHO) diets (12), and (ii), that HF diets elevate blood cholesterol levels while low-fat (LF) diets lower blood cholesterol levels (9–11). The first hypothesis may well be right, but one has to eat excess calories (i.e., take in more calories than expended daily) to gain weight regardless of the character of the diet, HF, LF, etc. The second hypothesis remains to be proven (11,12).

In most studies reporting the effects of HF and LF diets on human plasma FA and lipoprotein levels, the design involved adding to the diet an oil that had a fatty acid composition (FAC) very different from the FAC of the control diet

Copyright © 1995 by AOCS Press

(13–20). Thus, it is difficult to determine if simply reducing the fat content of the diet without changing the dietary FAC changes the tissue FAC or alters plasma lipid levels. Furthermore, people eat natural and processed food as complex mixtures that vary day to day and year to year. They rarely eat pure fats or oils, and certainly not continuously for weeks. In most previous studies (13–20) that have examined this issue, the FAC of the diet was changed markedly as well as the amount of calories from fat; thus, the experimental diets had different ratios of saturated and unsaturated FA in them. This confounds the interpretation of the results; one does not know if the changes observed are the result of changes in the caloric intake or changes in the type of FA in the diet. In an effort to clarify this point, we fed LF (22 en% fat) and HF (39 en% fat) diets to healthy volunteers for 50 d. The diets had FAC that were identical despite the intentional differences in the fat content in the diet.

MATERIALS AND METHODS

Materials. All natural foods were purchased at local food markets. FA standards were purchased from Nu-Chek-Prep (Elysian, MN). Organic solvents were obtained from Baxter Scientific, Burdick and Jackson Brand (McGaw Park, IL). The reagents for the enzymatic determination of plasma cholesterol and triglycerides were purchased from Sigma Diagnostics (St. Louis, MO).

Subjects. The volunteers were recruited from the West Coast, and consisted of men between the ages of 20 and 35. The physical characteristics of the eleven male volunteers (HNS-27) who completed the study were age (years), 32.9 ± 4.5; weight (kg), 72.9 ± 8.2; body mass index (kg/M^2), 23.1 ± 1.6; blood pressure systolic, 115.9 ± 9.5; blood pressure diastolic, 73.2 ± 7.8; smokers, none. Initially twelve volunteers were included in the study, but one was unable to complete the protocol. The volunteers were given complete physical examinations. Body weights had to be within −10 to +20 percent of ideal body weights using the Metropolitan Life Insurance Company tables (medium frame values from the 1983 edition). Evidence of existing illness or chronic disease was an exclusion criterion. Mild hypertension was not an exclusion criterion, but the group that was recruited tended to have blood pressures slightly below average for men in this age group. Smoking, excessive alcohol consumption, and evidence of narcotic abuse were also exclusionary.

Experimental design. All the volunteers were confined to the Nutrition Research Suite of the Western Human Nutrition Research Center (San Francisco, CA) for the duration of the study. As the subjects stay within the confines of the Nutrition Suite except for occasional supervised outings, they had no opportunity to consume any food except that provided by the Center. Thus, compliance with the protocol was, of necessity, 100%. In addition, all food intake was monitored, portions were weighed, and subjects were required to consume everything provided to them during their meals. (A rubber spatula was provided to ensure that all food was scraped from the plates and eaten.) Food spills were carefully monitored and recorded, and fluid intake, while *ad libitum,* was also measured precisely.

The protocol for this study was approved by the Institutional Review Boards of the University of California at Davis (Davis, CA) and the USDA (Washington, D.C.). A crossover design was used so that the subjects acted as their own controls. The subjects were fed a stabilization diet, containing 39% of calories from fat for 20 d. The HF diet had a macronutrient composition of 39 en% fat, 16 en% protein, and 45 en% CHO. On day 20 of the study, they were divided randomly into two groups: Group A remained on the HF diet (39% of calories from fat) for 50 d; Group B was placed on a LF diet (22% of calories from fat). The LF diet had a macronutrient composition of 22 en% fat, 16 en% protein, and 62 en% CHO. After 50 d, day 70 of the study, the groups switched diets for the remaining 50 d of the study. After 120 d, all the volunteers were discharged from the Nutrition Suite and returned to an *ad libitum* diet.

During the confinement period, there were blood draws on study days 2, 20, 45, 70, 95, and 120. As measured parameters on the intermediate blood draws (study days 45 and 95) showed insignificant differences from the endpoints (study days 70 and 120), only data obtained from the endpoint blood draws were used for statistical analysis. Statistical comparisons of the measured parameters were made using day 20 as the baseline values for each subject.

Diets. The diets consisted of natural foods. No dietary supplements were given. The macronutrient composition of the diets is given in Table 1. (A complete description of the diets, listing all the major and minor nutrients, is available upon request.) A seven-day menu cycle was used throughout the study. Proximate analysis was made on seven individual diet composite samples taken from each menu once during the study for both the stabilization diet and intervention diets. The results for the seven composite samples were averaged to find the actual composition of the diets. No alcohol was in-

TABLE 1
Composition of HNS-27 Diets, Proximal Analysis[a]

	Low-fat diets		High-fat diets	
	(% of total calories)			
	Measured	Target value	Measured	Target value
Macronutrient energy distribution				
Protein	15.9	16.0	15.7	16.0
Fat	22.2	20.0	38.7	40.0
Carbohydrate	61.9	64.0	45.7	44.0
Cholesterol content (mean, mg/day)	—	360	—	360
Fatty acid energy distribution				
Saturated	6.4	5	10.6	10
Monounsaturated	9.2	10	15.5	20
Polyunsaturated	6.6	5	12.6	10
P/S ratio	1.0	1.0	1.2	1.0

[a]P/S, polyunsaturated/saturated; HNS-27 diet.

TABLE 2
Fatty Acid Composition (wt%) of Experimental Diets

FAME[a]	High-fat diet (means ± SD)	Low-fat diet (means ± SD) (n = 7)	*P* values
12:0 (laurate)	0.9 ± 0.5	0.8 ± 0.4	
14:0 (myristate)	2.4 ± 0.8	2.2 ± 1.3	
15:0	0.3 ± 0.1	0.3 ± 0.1	
16:0 (palmitate)	14.9 ± 2.7	15.3 ± 2.8	
16:1n-9	0.7 ± 0.3	0.9 ± 0.3	
17:0	0.3 ± 0.1	0.3 ± 0.1	
18:1 DMA	0.3 ± 0.1	0.3 ± 0.1	
18:0 (stearate)	8.5 ± 1.4	8.7 ± 1.7	
18:1*t*, all isomers	5.2 ± 1.5	6.0 ± 1.4	
18:1n-9 (oleate)	32.1 ± 3.1	32.4 ± 3.7	
18:1n-7	1.6 ± 0.3	1.6 ± 0.2	
18:2*t* and 19:0	0.4 ± 0.2	0.6 ± 0.2	
18:2n-6 (linoleate)	22.9 ± 6.5	20.7 ± 6.3	
20:0 (arachidate)	0.6 ± 0.1	0.6 ± 0.1	
18:3n-3 (α-linolenate)	6.4 ± 4.8	7.3 ± 6.2	
20:1n-11	0.3 ± 0.1	0.3 ± 0.0	
22:0 (behenate)	0.3 ± 0.0	0.3 ± 0.1	
20:4n-6 (arachidonate)	0.2 ± 0.1	0.4 ± 0.1	$P < 0.001$
24:0 (lignocerate)	0.2 ± 0.0	0.2 ± 0.0	
Sum of minor components	0.7	0.2	
Total	99.0 ± 0.2	99.3 ± 0.5	
Unknowns	1.0 ± 0.2	0.7 ± 0.5	

[a]Fatty acid methyl esters; DMA, dimethyl acetate.

cluded in these diets. The nutrient composition of the diets was calculated from a computerized nutrient data bank using the data from USDA Handbook 8 (21), and adjusted to provide at least the recommended daily allowance for known essential nutrients (22).

Table 2 gives the measured FAC of both diets. The ratios of all the major FA were intended to be held constant in these diets. Inspection of Table 2 indicates that all the major FA were very similar although the level of linoleic acid was slightly higher in the HF diet, 23% in the HF diet vs. 21% in the LF diet. As the diets consisted of only natural food, the initial FAC calculated from food composition tables matched very well with the analyses obtained on the actual diet. At the beginning of the study, each participant's caloric intake was estimated, and the caloric content of the meals was calculated to maintain current body weight. As the estimates were subjected to error, the participants were weighed every day, and the caloric value of their meals adjusted occasionally, if necessary, to maintain their weight within the limits of the study. Thus, the average weights of the participants did not vary significantly during the 120 d of the study. If all the intakes for each volunteer were averaged during the entire study, the mean energy intake was approximately 2800 Kcal/d.

Measurements. Blood was drawn between 7:00 and 8:00 a.m. after an overnight fast of at least 12 h. Blood was drawn into vacutainers with appropriate anticoagulants using a Teflon catheter (Angiocath; Desert Medical, Sandy, UT) for FA analysis, or syringes containing 1 mL of citrate solution for platelet studies. Platelet-rich plasma was prepared by low-speed centrifugation for 10 min at 100 × *g*. Platelet-poor plasma was prepared by respinning the blood after removal of the platelet-rich plasma for 15 min at 400 × *g*. Red cells for FA analyses were prepared from the residue by resuspending the cells in 10 mL of phosphate buffered saline and recentrifuging the cells at 400 × *g* for 15 min three times. The supernate was discarded. The red cells were frozen at −70°C until their lipids could be extracted.

Triglyceride and cholesterol determinations. Plasma was analyzed for triglycerides and cholesterol in a Cobas-Faras centrifugal analyzer (Roche Diagnostic Systems, Montclair, NJ) using automated enzymatic methods as described by McGowan *et al.* (23) and Allain *et al.* (24).

Lipid extraction and FA analysis. Tissues were extracted by the procedures described by Nelson (25,26) using chloroform/methanol (2:1, vol/vol). Samples were then transmethylated with methanolic HCl (7%, w/w) by procedures described elsewhere (26,27). When the total lipid extract was transmethylated, the impurities, extracted into the hexane phase after termination of the reaction, were removed by thin-layer chromatography as described by Nelson (25). Because the amount of material available from human platelets for FA analysis was limited FA methyl esters were obtained by direct transmethylation of the intact tissue without prior solvent extraction as described in an earlier publication (28). The conditions of the capillary gas–liquid chromatography (GLC) have been described previously (29). Briefly, the column was a 30 m × 0.025 mm fused quartz column coated with SP-2340 purchased from Supelco, Inc. (Bellefonte, PA). The GLC data were processed with Hewlett-Packard ChemStation software (Palo Alto, CA) running on a desktop computer. The gas chromatograph was a Hewlett-Packard Model 5890a. Data were reprocessed and combined using Excel 5.0 obtained from Microsoft Corporation (Bellevue, WA).

Statistical analysis. Lipid and FA data were analyzed with a single tailed, paired *t*-test, using SigmaStat (Jandel Scientific, San Rafael, CA) statistical program for IBM compatible computer. Analysis of variance statistical analyses were performed with a desktop computer using the PC SAS data system, version 6.08, obtained under license from the SAS Institute, PC-SAS, Version 6.08 (SAS PC Manual, Version 6.08, 1993; SAS Institute, Chapel Hill, NC). Both statistical treatments yielded essentially the same results. The power of this study to detect a significant difference at the $P < 0.05$ level was 90% with eleven subjects (n = 11) assuming a difference of 20% in the means of lipid parameters and a standard deviation of ±20% of the mean value.

RESULTS

The measured and theoretical values on the macronutrient composition of the LF and HF diets were extremely close (Table 1). The only significant difference between the fatty acid composition of the LF and HF diets was the slightly larger amount of arachidonic acid (AA) in the LF diet compared to the HF diet (Table 2). The linoleic acid content of the

HF diet was about 2% greater than the amount in the LF diet. This did not reach statistical significance, however, due to the large standard deviation between amounts in the individual menu plans in the seven-day menu rotation. This is a statistical quirk of the analysis. The difference in the amount of AA in the two diet regimes probably had little physiological significance.

The FAC of the subjects' plasma, red blood cells, and platelets are given in Tables 3 through 5. In each tissue, FAC remained similar on each diet. There is a small but constant elevation in the linoleic acid content of the blood from subjects consuming the HF diet. In the plasma and platelets, the linoleic acid content of the diet was significantly different at the $P < 0.05$ level, and equivalent to the amount of excess linoleic acid in the HF diet. Other differences were detected in the amount of specific FA in the plasma and platelets. These differences which were statistically significant usually involved FA that were present in small amounts. The physiological significance of these small differences, if any, is unknown. It also is possible that in several of these cases the statistical significance is artifactual.

In the red blood cell samples, the situation appears more complex. While plasma and platelet FA turn over rapidly in the body, 1–2 and 8–16 d, respectively, red cell FAC shows a consistency related to the 120-d turnover of the red cells in the circulation. During the 50-d intervention, we observed that the red cell linoleic acid level was reduced by the LF diet and increased by the HF diet, consistent with the difference in the FAC of the two diets. Besides this change in linoleic acid levels, red cells exhibited changes in stearate, α-linolenic acid, and dihomo-γ-linolenic acid, and AA. This suggests that the linoleic acid elongation and desaturation pathways were influenced by the amount of fat in the diet.

Table 6 lists the plasma cholesterol, triglycerides, high density lipoprotein (HDL)-cholesterol, and low density lipoprotein (LDL)-cholesterol levels before and after the subjects consumed the LF and HF diets. Also shown are their lipid values prior to the start of the study and after three weeks on the stabilization diet. Although the total cholesterol, HDL-cholesterol, and LDL-cholesterol levels are slightly increased when the subjects consumed the HF diet compared to the values obtained after they consumed the LF diet, the increase was not statistically significant.

The triglycerides' values did change significantly ($P < 0.002$), rising about 25 mg/dl when the subjects were consuming the LF diet compared to the period when they consumed the HF diet. Thus, although the cholesterol levels in the vari-

TABLE 3
Plasma Fatty Acid Composition (wt%)

FAME[a]	High-fat diet (means ± SD)	Low-fat diet (means ± SD)	*P* values
14:0 (myristate)	0.55 ± 0.12	0.64 ± 0.27	0.23
16:0 DMA	0.29 ± 0.07	0.16 ± 0.12	
16:0 (palmitate)	20.15 ± 1.27	21.9 ± 1.83	
16:1*t*	0.09 ± 0.11	0.14 ± 0.16	
16:1n-9	0.62 ± 0.31	1.13 ± 0.45	0.0014
16:1n-7	0.37 ± 0.24	0.33 ± 0.18	
17:0	0.39 ± 0.03	0.40 ± 0.04	
18:0 DMA	0.23 ± 0.06	0.23 ± 0.04	
18:1 DMA	0.51 ± 0.29	0.46 ± 0.26	
18:0 (stearate)	.21 ± 0.93	7.97 ± 0.97	
18:1*t*	1.59 ± 0.30	1.62 ± 0.25	
18:1n-9 (oleate)	16.17 ± 2.16	17.62 ± 1.91	
18:1n-7	1.33 ± 0.12	1.57 ± 0.15	0.00002
18:1n-5	0.65 ± 0.06	0.78 ± 0.12	0.0097
18:2*tt* and 19:0	0.32 ± 0.14	0.33 ± 0.08	
18:2n-6 (linoleate)	31.84 ± 2.21	27.17 ± 2.24	0.003
18:3n-3 (α-linolenate)	0.28 ± 0.05	0.34 ± 0.08	
20:0 (arachidate)	0.45 ± 0.05	0.41 ± 0.05	
18:3n-3 (linolenate)	1.34 ± 0.42	1.11 ± 0.30	
20:3n-6	0.97 ± 0.18	1.2 ± 0.19	0.0004
22:0 (behenate)	0.96 ± 0.13	0.8 ± 0.12	0.002
20:4n-6 (arachidonate)	6.32 ± 1.06	7.08 ± 1.73	
22:2n-6	0.37 ± 0.24	0.25 ± 0.23	
20:5n-3 (EPA)	0.22 ± 0.21	0.21 ± 0.20	
24:0 (lignocerate)	0.86 ± 0.11	0.73 ± 0.11	0.004
24:1n-9 (nervonate)	1.17 ± 0.17	1.19 ± 0.24	
22:5n-3 (DPA)	0.34 ± 0.06	0.42 ± 0.07	0.0004
22:6n-3 (DHA)	0.88 ± 0.11	1.11 ± 0.20	0.0004
Total	99.06 ± 0.52	99.08 ± 0.45	
Unknown	0.94 ± 0.52	0.92 ± 0.45	

[a]See Table 2 for abbreviations; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

TABLE 4
Platelet Fatty Acid Composition (wt%)[a]

FAME	High-fat diet (means ± SD)	Low-fat diet (means ± SD)	*P* values
14:0 (myristate)	0.29 ± 0.09	0.24 ± 0.04	
16:0 DMA	1.75 ± 0.23	1.92 ± 0.25	
16:0 (palmitate)	11.86 ± 0.54	12.24 ± 0.67	
16:1n-9	0.16 ± 0.06	0.19 ± 0.04	
17:0	0.28 ± 0.01	0.28 ± 0.02	
18:0 DMA	3.05 ± 0.34	3.02 ± 0.24	
18:1 DMA	1.11 ± 0.21	1.19 ± 0.17	
18:0 (stearate)	14.7 ± 0.47	14.60 ± 0.95	
18:1*t*	1.47 ± 0.07	1.49 ± 0.18	
18:1n-9 (oleate)	11.89 ± 0.62	11.96 ± 0.6	
18:1n-7	0.77 ± 0.07	0.72 ± 0.26	
18:1n-5	0.56 ± 0.04	0.54 ± 0.2	
18:2*tt* and 19:0	0.24 ± 0.06	0.23 ± 0.04	
18:2n-6 (linoleate)	8.01 ± 1.35	6.69 ± 0.78	0.02
20:0 (arachidate)	1.73 ± 0.19	1.53 ± 0.14	0.02
18:3n-3 (α-linolenate)	0.41 ± 0.14	0.51 ± 0.64	
20:1n-11	0.6 ± 0.09	0.57 ± 0.07	
20:3n-6 (homo-γ-linolenate)	1.17 ± 0.25	1.21 ± 0.28	
22:0 (behenate)	2.24 ± 0.25	2.00 ± 0.23	0.04
20:4n-6 (arachidonate)	25.51 ± 1.32	25.87 ± 1.05	
22:1n-6	0.3 ± 0.06	0.19 ± 0.10	0.008
20:5n-3 (EPA)	0.35 ± 0.19	0.46 ± 0.09	
24:0 (lignocerate)	1.15 ± 0.16	1.01 ± 0.16	
24:1n-11	2.1 ± 0.27	1.89 ± 0.69	
24:1n-9 (nervonate)	1.56 ± 0.26	1.65 ± 0.26	
22:4n-3	0.51 ± 0.4	0.33 ± 0.21	
22:5n-3 (DPA)	1.94 ± 0.3	1.84 ± 0.23	
22:6n-3 (DHA)	1.74 ± 0.38	2.01 ± 0.39	
Total	98.05 ± 0.69	97.99 ± 0.72	
Unknown	1.95 ± 0.69	2.01 ± 0.72	

[a]See Tables 2 and 3 for abbreviations.

TABLE 5
Red Blood Cell Fatty Acid Composition (wt%)[a]

FAME	High-fat diet (means ± SD)	Low-fat diet (means ± SD)	*P* values
14:0 (myristate)	0.20 ± 0.05	0.19 ± 0.03	
16:0 DMA	1.51 ± 0.17	1.62 ± 0.11	
16:0 (palmitate)	16.57 ± 1.57	16.16 ± 0.38	
16:1n-9	0.35 ± 0.04	0.33 ± 0.02	
17:0	2.87 ± 0.46	3.04 ± 0.19	
18:0 DMA	0.35 ± 0.05	0.38 ± 0.04	
18:1n-7 DMA	1.64 ± 0.28	1.75 ± 0.22	
18:0 (stearate)	10.25 ± 0.58	9.74 ± 0.36	0.01
18:1n-7*t*	1.11 ± 0.07	1.06 ± 0.1	
18:1n-9 (oleate)	9.51 ± 0.82	9.11 ± 0.8	
18:1n-7	0.85 ± 0.06	0.88 ± 0.08	
18:1n-5	0.44 ± 0.05	0.47 ± 0.07	
18:2*tt* and 19:0	0.11 ± 0.02	0.11 ± 0.01	
18:2n-6 (linoleate)	9.99 ± 0.61	8.37 ± 0.46	0.000004
20:0 (arachidate)	0.61 ± 0.11	0.54 ± 0.05	
18:3n-3 (α-linolenate)	0.30 ± 0.05	0.25 ± 0.04	0.0005
20:3n-6 (homo-γ-linolenate)	1.12 ± 0.23	1.34 ± 0.24	0.00003
22:0 (behenate)	2.22 ± 0.47	2 ± 0.19	
20:4n-6 (arachidonate)	12.11 ± 2.34	13.85 ± 0.47	0.04
23:0	0.47 ± 0.1	0.42 ± 0.04	
20:5n-3 (EPA)	0.52 ± 0.14	0.61 ± 0.11	
24:0 (lignocerate)	6.54 ± 1.56	5.94 ± 0.59	
24:4n-6	3.12 ± 0.73	3.49 ± 0.34	
24:1n-9 (nervonate)	6.38 ± 1.32	6.03 ± 0.5	
22:4n-3	0.56 ± 0.15	0.74 ± 0.13	0.007
24:2n-6	1.05 ± 0.16	1.05 ± 0.09	
22:5n-3	2.6 ± 0.69	2.99 ± 0.3	
22:6n-3 (DHA)	4.02 ± 1.3	4.87 ± 0.81	
26:0	0.44 ± 0.11	0.41 ± 0.05	
Total	99.04 ± 0.16	98.95 ± 0.13	
Unknown	0.96 ± 0.16	1.05 ± 0.13	

[a]See Tables 2 and 3 for abbreviations.

ous lipoprotein fractions were not altered significantly, there was a redistribution of the lipoprotein spectrum during the two diet periods. The increase in the plasma triglyceride level indicated an increase in the plasma very low density lipoprotein (VLDL) level and a decrease in both the plasma HDL and LDL levels. Thus, the total cholesterol level remained constant because the increase in plasma VLDL cholesterol compensated for the reduction in HDL-cholesterol and LDL-cholesterol. Because of individual variations, the standard deviation was large enough to prevent these values from reaching statistical significance.

DISCUSSION

It has almost been dogma for the last forty years in the field of dietary fat effects on blood lipids that HF diets will raise blood cholesterol levels while LF diets will lower blood cholesterol (1–4). A problem one encounters when examining the scientific basis for this concept is that the FAC of LF and HF diets was rarely the same. The Keys *et al.* (5) and Hegsted *et al.* (6) formulas for the effect of dietary fat on blood cholesterol levels were published thirty years ago. Those equations say nothing about the total percentage of fat calories in the diet, but they imply that HF diets will raise blood cholesterol because they relate grams of fat ingested to blood cholesterol level, positively for saturated FA, except stearic (7,8), and negatively for polyunsaturated fat (n-3 FA are ignored). Dietary cholesterol has only a small influence on blood cholesterol levels. If one uses the Keys equation (5) or the Hegsted equation (6) to calculate the change in blood cholesterol that one would expect with the protocol used in this study, there should have been an average difference between the HF and LF diets of −23 mg/dl. The subjects' LF diet cholesterol level was 173 mg, and should have risen to 196 mg/dl if the Keys *et al.* (5) and Hegsted *et al.* (6) equations were an appropriate explanation for the physiological response to changes in dietary fat intakers. The observed total cholesterol value when the participants consumed the HF was 177 mg/dl, not significantly different from the value obtained when they consumed the LF diet.

There are several possible reasons that the Keys *et al.* (5) and Hegsted *et al.* (6) equations do not hold in this particular experiment: (i) The equations were developed using large population data bases (11,20). Here the sample number is too small to detect the effect. This answer is unlikely and unsatisfactory because the conditions of this experiment were carefully controlled and the statistical power of the protocol was excellent.

(ii) The average cholesterol level in the subjects was considerably below that in the European and American populations used to develop the Keys *et al.* (5) and Hegsted *et al.* (6)

TABLE 6
High- and Low-Fat Diets, Blood Cholesterol, Triglycerides, and Lipoprotein Values

Period	Diet	Total cholesterol mean ± SD	Triglycerides mean ± SD	HDL-cholesterol mean ± SD	LDL-cholesterol mean ± SD
Entry	*Ad libitum*	176.3 ± 33.1	85.8 ± 28.4	46.3 ± 14.0	112.8 ± 26.8
Stabilization	High-fat	172.5 ± 30.3	75.3 ± 46.4	44.8 ± 11.6	112.6 ± 21.9
Intervention	Low-fat	173.2 ± 27.3	91.5 ± 38.0	40.5 ± 12.4	114.5 ± 21.3
Intervention	High-fat	176.9 ± 32.9	66.4 ± 41.7	43.2 ± 13.4	119.5 ± 24.3
Paired *t*-test, *P* values[a]		0.425	0.002	0.258	0.238

[a]The *t*-test compares only the values at the end of the high- or low-fat diets with the values obtained at the end of the stabilization period, study day 20. Groups A and B values were taken at study day 70 and day 120, depending on the leg of the intervention diet for the each group; HDL, high density lipoprotein; LDL, low density lipoprotein.

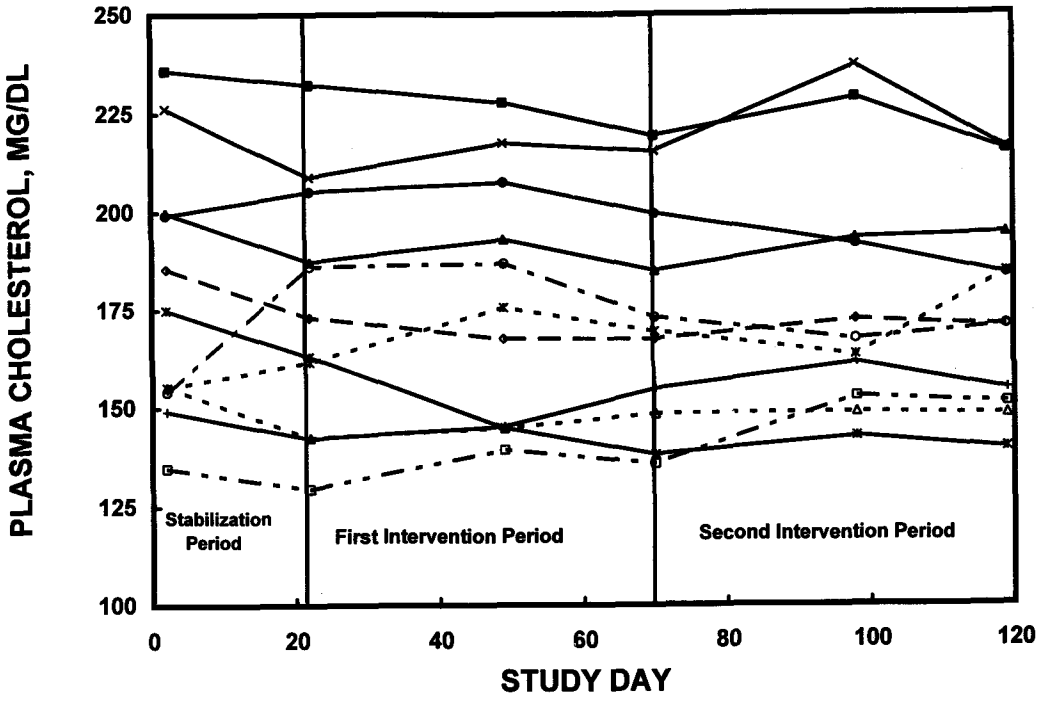


FIG. 1. The individual plasma total cholesterol values for the all participants during this study. The various study periods are shown on the figure. Separate symbols are used for each subject. Solid lines represent members of Group A who received the high-fat diet in the first intervention period while the dotted lines represent members of Group B who received the high-fat diet in the second intervention period.

equations, and it is impossible to lower blood cholesterol below a certain point by dietary manipulation alone (22). If subjects with higher mean cholesterol values were studied, the cholesterol levels would have dropped as predicted by the equations. We think not. The average cholesterol level of the group was 176 mg/dl on entry into the study. It was 177 mg/dl on the HF diet and 173 mg/dl on the LF diet. It did not go up or down on the intervention diets. Also, a blood cholesterol level of 177 mg/dl is not exceptionally low. It is above the mean level in Japan and several other non-European cultures (30,31) and about the level observed in vegetarians in the United States (32). Furthermore, if one looks at the plasma cholesterol levels of the individuals in this study, the values ranged from 133 to 240 mg/dl, yet none showed any significant drop from entry level on the LF diet nor any marked rise on the HF diet (Fig. 1).

(iii) The Keys *et al.* (5) and Hegsted *et al.* (6) equations were developed using data from populations which consumed diets in which the polyunsaturated/saturated ratio varied from 0.2 to 0.4, not the 1.0 found in the diets used in this study. If the P/S ratios in the diets used in this study were in the same range as that in the general American or European populations, the equations would have predicted the change in blood cholesterol level correctly. Perhaps, but that is just another way to restate our hypothesis: it is the ratio of the various FA in the diets that determines the changes in blood cholesterol levels upon changes in the dietary fat intake, not the percentage of calories from fat, either saturated or unsaturated. Hayes *et al.* (33–35) have suggested that if polyunsaturated fat in the diet makes up more than 6% of calories, it is not hypocholesterolemic. In this study, polyunsaturated fat accounted for 13% of calories in the HF diet and 7% of calories in the LF diet.

(iv) There are responders and nonresponders to dietary fat. This study only included nonresponders. That is highly unlikely as the subjects were randomly selected from the general population and were not selected on the basis of their cholesterol values nor tested for a response to dietary fat.

(v) The ratio of saturated to monounsaturated to polyunsaturated fat in the diet will determine the change in plasma cholesterol level in response to changes in dietary fat intakes, not the actual level of fat in the diet (i.e., the percentage amount of fat calories in the diet). We feel that this is the correct interpretation of the results from this study.

Obviously, if fat calories are significantly reduced in the diet, they must be replaced with another macronutrient, such as CHO. If the amount of CHO in the diet is increased, a temporary or perhaps permanent increase in the triglyceride levels in plasma will occur (36–39). While it is also possible that the observed increase in plasma triglycerides here, and also reported by many other investigators (37–39), was transient (40), a report by West *et al.* (41) suggests that high CHO diets induce a permanent elevation in plasma triglycerides and, consequently, in the VLDL levels in humans. It is still controversial whether increases in VLDL have any adverse health effects (42–44).

It also appears as if increased levels of saturated fat may cause an increase in HDL levels (12,45–47). But it is not clear whether this increase is due to the decrease in triglyceride levels found in low CHO diets, as there is an inverse relationship between plasma triglyceride levels and HDL levels (12). LDL-cholesterol levels, the cholesterol value most associated with heart disease risk (44), are unaffected by the percentage of fat calories in the diet. This has wide dietary implications for individuals trying to lower their plasma LDL levels as well as for health-care providers advising individuals on dietary modification as related to cardiovascular risk and health.

As the FAC of both intervention diets was held constant, there were few changes in the FAC of the three tissues examined here, plasma, platelets, and red cells. It was interesting, however, that the small difference (2%) between the linoleic acid content in the two diets was easily detectable in all of these tissues. This would seem to indicate that the tissue levels of essential fatty acids are determined primarily by diet and not by a homeostatic control in the tissue or organ. The other differences in the FAC between the tissue when the subjects consumed either the LF or HF diets may be due to small differences in the FAC of the two diets, or are artifacts of the analytical methods when FA are present in small amounts in the tissues. The differences observed in the FAC of the red cells, particularly dihomo-γ-linoleic and AA, may be real and related to the metabolism of linoleic acid. There is an inverse relationship between linoleic and AA levels in most tissues (48,49).

While we consider that the increased linoleic acid content of the plasma, platelets, and red blood cells reflects the 2% difference in the linoleic acid content between the LF and HF diets, this is only one possible explanation. Other factors that could account for the differences could be related to differences in metabolism between subjects consuming high CHO and LF diets vs. subjects consuming moderate CHO, HF diets. As this study did not have an isotopic tracer included in its design, there is no way in which this question can be answered definitively.

There are several caveats that must be attached to these results. This study involved only eleven subjects, between the ages of 20 and 37, who were all male, normal lipemic, and in excellent health who did not smoke or ingest alcohol (or other drugs) during this study. All environmental conditions were kept constant as the volunteers were confined to a metabolic unit for the duration of the study. Whether the results of this study can be extrapolated to free-living, hyperlipidemic subjects, other age groups or gender remains to be determined by further experiments. Still, it is apparent that one must be cautious in predicting changes in blood cholesterol from lowering total dietary fat intakes or applying the Keys *et al.* (5) and Hegsted *et al.* (6) equations to individuals when recommending dietary modifications with respect to fat intakes. The relationship of dietary fat intakes to blood cholesterol levels is very complex, so much of the information gleaned from human feeding studies using artificial mixtures of oils and diets must be viewed with skepticism when applied to individuals with dyslipoproteinemias.

ACKNOWLEDGMENTS

The authors wish to acknowledge the help of the staff at our Center. We are especially indebted to the Metabolic Research Unit staff, without whose help this study could not have been conducted, including the nursing staff who drew the blood and the dietetics staff who prepared the food. We also wish to thank the Bioanalytical Lab staff who did the plasma cholesterol and triglyceride analyses. We appreciate the help of Giovanni Bartolini with maintenance of the gas-chromatograph.

REFERENCES

1. Kritchevsky, D., Moyer, A.W., Tesar, W.C., Logan, J.B., Brown, R.A., Davies, M.C., and Cox, H.R. (1954) Effect of Cholesterol Vehicle in Experimental Atherosclerosis, *Am. J. Physiol. 178*, 30–32.
2. Kritchevsky, D., Moyer, A.W., Tesar, W.C., McCandless, R.F., Logan, J.B., Brown, R.A., and Englert, M. (1956) Cholesterol Vehicle in Experimental Atherosclerosis II. Effect of Unsaturation, *Am. J. Physiol. 185*, 279–280.
3. Groen, J. (1958) Present Status of Knowledge of the Various Factors in the Etiology of Atherosclerotic Heart Disease, *Netherlands Milk Dairy J. 12*, 282–338.
4. Ahrens, E.H., Jr., Hirsch, J., Insull, W., Jr., Tsaltas, T.T., Blomstrand, R., and Peterson, M.L. (1957) The Influence of Dietary Fats on Serum Lipid Levels in Man, *Lancet 1*, 943–953.
5. Keys, A., Anderson, J.T., and Grande, F. (1965) Serum Cholesterol Response to Changes in the Diet. IV, Particular Fatty Acids in the Diet, *Metabolism 14*, 776–787.
6. Hegsted, D.M., McGandy, R.B., Myers, M.L., and Stare, F.J. (1965) Quantitative Effect of Dietary Fat on Serum Cholesterol in Man, *Am. J. Clin. Nutr. 17*, 281–295.
7. Bonanome, A., and Grundy, S.M. (1988) Effect of Dietary Stearic Acid on Plasma Cholesterol and Lipoprotein Levels, *N. Engl. J. Med. 318*, 1244–1248.
8. Dougherty, R.M., Allman, M.A., and Iacono, J.M. (1995) Effect of Diets Containing High or Low Amounts of Stearic Acid on Plasma Lipoprotein Fractions and Fecal Fatty Acid Excretion of Men, *Am. J. Clin. Nutr. 61*:1120–1128.
9. Consensus Conference: Lowering Blood Cholesterol to Prevent Heart Disease (1985), *JAMA 253*, 2080–2088.
10. Beynen, A.C., and Katan, M.B. (1989) Impact of Dietary Cholesterol and Fatty Acids on Serum Lipids and Lipoproteins in Man, in *The Role of Fats in Human Nutrition. London* (Vergroesen, A.J., and Crawford, M.A., eds.) Academic Press Ltd., pp. 237–286.
11. Erkelens, D.W. (1989) Cholesterol Consensus in the Netherlands, *Eur. J. Clin. Nutr. 43*, 89–96.
12. Mensink, R.P., and Katan, M.B. (1993) Dietary Fatty Acids and Serum Lipids, *Arteriosclerosis Thrombosis 12*, 911–919.
13. Brussaard, J.H., Katan, M.B., Groot, P.H.E., Havekes, L.M., and Hautvast, J.G.A.H. (1982) Serum Lipoproteins of Healthy Persons Fed a Low-Fat Diet or a Polyunsaturated Fat Diet for Three Months, *Atherosclerosis 42*, 205–219.
14. Chenoweth, W., Ullmann, M., Simpson, R., and Leveille, G. (1981) Influence of Dietary Cholesterol and Fat on Serum Lipids in Men, *J. Nutr. 111*, 2069–2080.
15. Laine, D.C., Snodgrass, C.M., Dawson, E.A., Ener, M.A., Kuba, K., and Frantz, I.D. (1982) Lightly Hydrogenated Soy Oil Versus Other Vegetable Oils as a Lipid-Lowering Dietary Constituent, *Am. J. Clin. Nutr. 35*, 683–690.
16. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) The Comparative Reductions of the Plasma Lipids and Lipoproteins by Dietary Polyunsaturated Fats: Salmon Oil Versus Vegetable Oils, *Metabolism 32*, 179–184.

17. Grundy, S.M., Nix, D., Whelan, M.F., and Franklin, L. (1986) Comparison of Three Cholesterol-Lowering Diets in Normolipidemic Men, *JAMA 256,* 2351–2355.
18. Rassias, G., Kestin, M., and Nestel, P.J. (1991) Linoleic Acid Lowers LDL Cholesterol Without a Proportionate Displacement of Saturated Fatty Acid, *Eur. J. Clin. Nutr. 45,* 315–320.
19. Woolett, L.A., Spady, D.K., and Dietschy, J.M. (1992) Saturated and Unsaturated Fatty Acids Independently Regulate Low Density Lipoprotein Receptor Activity and Production Rate, *J. Lipid Res. 33,* 77–88.
20. Hegsted, D.M., Ausman, L.M., Johnson, J.A., and Dallard, G.E. (1993) Dietary Fat and Serum Lipids, *Am. J. Clin. Nutr. 57,* 875–883.
21. U.S. Department of Agriculture, *Composition of Foods,* Revised, Agriculture Handbook No. 8.1-8.14. U.S. Government Printing Office, Washington, D.C., 1987.
22. National Research Council, Recommended Dietary Allowances, 10th edn., Washington, D.C., National Academy Press, 1989.
23. McGowan, M.W., Artiss, J.D., Strandberg, D.R., and Zak, B. (1983) A Peroxide-Coupled Method for the Colorimetric Determination of Serum Triglycerides, *Clin. Chem. 29,* 538–548.
24. Allain, C.A., Poon, L.S., Chan, C.G.S., Richmond, W., and Fu, P.C. (1974) Enzymatic Determination of Total Serum Cholesterol, *Clin. Chem. 20,* 470–479.
25. Nelson, G.J. (1972) Handling, Extraction and Storage of Blood Samples, in *Blood Lipids and Lipoproteins* (Nelson, G.J., ed.) pp. 3–24, Wiley-Interscience, New York.
26. Nelson, G.J., (1975) The Isolation and Purification of Lipids from Animal Tissue, in *Analysis of Lipids and Lipoproteins* (Perkins, E.G., ed.) pp. 1–22, American Oil Chemists' Society. Champaign, IL.
27. Nelson, G.J., Kelley, D.S., and Hunt, J.E. (1986) Effect of Nutritional Status on the Fatty Acid Composition of Rat Liver and Cultured Hepatocytes, *Lipids 21,* 454–459.
28. Nelson, G.J., Kelley, D.S., Schmidt, P.C., and Serrato, C.M. (1987) The Effect of Fat-Free, Saturated and Polyunsaturated Fat Diets on Rat Liver and Plasma, *Lipids 22,* 88–94.
29. Nelson, G.J., Kelley, D.S., Schmidt, P.C., and Serrato, C.M. (1987) The Influence of Dietary Fat on the Lipogenic Activity and Fatty Acid Composition of Rat White Adipose Tissue, *Lipids 22,* 338–334.
30. Keys, A. (1957) Diet and the Epidemiology of Coronary Heart Disease, *J. Am. Med. Assoc. 164,* 1912–1919.
31. Antonis, A., and Bersohn, I. (1962) The Influence of Diet on Serum Lipids in South African White and Bantu Prisoners, *Am. J. Clin. Nutr. 10,* 484–499.
32. Walden, R.T., Schaefer, L.E., Lemon, F.R., Sunshine, A., and Wynder, E.L. (1964) Effect of Environment on the Serum Cholesterol-Triglyceride Distribution Among Seventh-day Adventists, *Am. J. Med. 36,* 269–276.
33. Hayes, K.C., Pronczuk, A., Lindsev, S., and Diersen-Schade, D. (1991) Dietary Saturated Fatty Acids (12:0, 14:0, 16:0) Differ in Their Impact on Plasma Cholesterol and Lipoproteins in Nonhuman Primates, *Am. J. Clin. Nutr. 53,* 491–498.
34. Khosla, P., and Hayes, K.C. (1992) Comparison Between the Effects of Dietary Saturated (16:0), Monounsaturated (18:0), and Polyunsaturated (18:2) Fatty Acids on Plasma Lipoprotein Metabolism in Cebus and Rhesus Monkeys Fed Cholesterol-Free Diets, *Am. J. Clin. Nutr. 55,* 51–62.
35. Hayes, K.C., and Khosla, P. (1992) Dietary Fatty Acid Thresholds, and Cholesterolemia, *FASEB J. 6,* 2600–2607.
36. Mensink, R.P., and Katan, M.B. (1987) Effect of Monounsaturated Fatty Acids Versus Complex Carbohydrates on High-Density Lipoproteins in Healthy Men and Women, *Lancet i,* 122–124.
37. Grundy, S.M., Florentin, L., Nix, D., and Whelan, M.F. (1988) Comparison of Monounsaturated Fatty Acids and Carbohydrates for Reducing Raised Levels of Plasma Cholesterol in Man, *Am. J. Clin. Nutr. 47,* 965–969.
38. Thompson, P.D., Cullinane, E.M., Eshelam, R., Kantor, M.A., and Herbert, P.N. (1984) The Effects of High-Carbohydrate and High-Fat Diets on the Serum Lipid and Lipoprotein Concentrations of Endurance Athletes, *Metabolism 33,* 1003–1010.
39. Szepesi, B. (1992) Interaction of Dietary Fatty Acids, Carbohydrates, and Lipids on Carbohydrate Metabolism, in *Fatty Acids in Foods and Their Health Implications* (Chow, C.K., ed.) pp. 473–499, Marcel Dekker, New York.
40. Hegsted, D.M. (1991) Dietary Fatty Acids, Serum Cholesterol and Coronary Heart Disease, in *Health Effects of Dietary Fatty Acids* (Nelson, G.J., ed.) pp. 50–68, AOCS Press, Champaign.
41. West, C.E., Sullivan, D.R., Katan, M.B., Halferkamps, I.L., and van der Torre, H.W. (1990) Boys from Populations with High Carbohydrate Intake Have Fasting Triglycerides Than Boys from Populations with High Fat Intake, *Am. J. Epidemiol. 131,* 271–282.
42. Austin, M.A. (1989) Plasma Triglycerides as a Risk Factor for Coronary Heart Disease: the Epidemiological Evidence and Beyond, *Am. J. Epidemiol. 129,* 249–259.
43. Assman, G., and Schulte, H. (1992) Role of Triglycerides in Coronary Heart Disease: Lesion from the Prospective Cardiovascular Munster Study, *Am. J. Cardiol. 70,* 10H–13H.
44. Havel, R.J., and Rapaport, E. (1995) Management of Primary Hyperlipidemia, *N. Eng. J. Med. 332,* 1491–1498.
45. Shepherd, J., Packard, C.J., Grundy, S.M., Yeshurun, D., Gotto, A.M. Jr., and Taunton, O.D. (1980) Effects of Saturated and Polyunsaturated Fat Diets on the Chemical Composition and Metabolism of Low Density Lipoproteins in Man, *J. Lipid Res. 21,* 91–99.
46. Mattson, F.H., and Grundy, S.M. (1985) Comparison of Effects of Dietary Saturated, Monounsaturated, and Polyunsaturated Fatty Acids on Plasma Lipids and Lipoproteins in Man, *J. Lipid Res. 26,* 194–202.
47. Mensink, R.P., and Katan, M.B. (1989) Effect of a Diet Enriched with Monounsaturated or Polyunsaturated Fatty Acids on Levels of Low-Density and High-Density Lipoprotein Cholesterol Levels in Healthy Women and Men, *N. Engl. J. Med. 321,* 436–441.
48. Phinney, P.D., Odin, R.S., Johnson, S.B., and Holman, R.T. (1990) Reduced Arachidonate in Serum Phospholipids and Cholesterol Esters Associated with Vegetarian Diets in Humans, *Am. J. Clin. Nutr. 51,* 385–392.
49. Nelson, G.J., Schmidt, P.C., and Corash, L. (1991) Effect of a Salmon Diet on Platelet Aggregation and the Fatty Acid Composition of Platelets, Plasma and Erythrocytes in Normal Adult Men, *Lipids 26,* 87–96.

[Received July 26, 1995, and in final revised form September 29, 1995; Revision accepted October 9, 1995]

Changes in Linoleic Acid Metabolism and Membrane Fatty Acids of LLC-PK Cells in Culture Induced by 5α-Cholestane-3β,5,6β-triol

M. Mahfouz, T. Smith and F.A. Kummerow*

Burnsides Research Laboratory, University of Illinois at Urbana, Illinois 61801
and Harlan E. Moore Heart Research Foundation, Champaign, Illinois 61820

ABSTRACT: *The aim of this study was to investigate the effect* of the oxysterol 5α-cholestane-3β,5,6β-triol (triol) on the metabolism of linoleic acid (18:2n-6) to arachidonic acid (20:4n-6) and on the cell membrane fatty acid composition. Porcine kidney cells were incubated in medium with or without 10 μg/mL of triol for 24 h, then incubated for 1, 6, or 12 h in a medium which contained 50 μM of either [^{14}C]linoleic acid or unlabeled linoleic acid. The cellular uptake of [^{14}C]linoleic acid was significantly higher in the triol-treated cells than in control cells. After 1- and 6-h incubations despite the increase of [^{14}C]linoleic acid pool size in the triol-treated cells, neither total n-6 polyunsaturated fatty acids (PUFA) metabolites nor arachidonic acid were increased in the triol-treated cells as compared to the control cells, but trienoic acids accumulated to a greater extent in the triol-treated cells. Therefore, the ratios of n-6 PUFA metabolites vs. pool size of linoleic acid and of tetraenoic acids vs. dienoic acids were significantly decreased in triol-treated cells as compared to the control cells. The cellular fatty acid composition also showed that linoleic acid percentage was significantly increased while arachidonic acid percentage was significantly decreased in the triol-treated cells, and that the accumulation of trienoic acids (18:3n-6 + 20:3n-6) observed from the [^{14}C]linoleic acid experiment was due solely to increased 20:3n-6 content. This latter finding indicates that a decrease of elongase activity by triol is unlikely. Our results also showed that the triol-treated cells had a lower level of free cholesterol but higher levels of phospholipid and triol in their membranes, suggesting that triol displaced free cholesterol from the cell membrane.

Lipids 30, 977–985 (1995).

Oxysterols (OS) have attracted attention because of their possible role in atherogenesis (1–4) as well as their involvement in the feedback regulation of cholesterol biosynthesis (5). In humans, OS are derived from the diet primarily as products of cholesterol autoxidation (6). High concentrations of OS are formed in certain processed food (7,8), and they are easily absorbed and transported in the blood (2). OS also can be produced endogenously (1).

Previous studies have provided evidence that OS such as 25-hydroxycholesterol and triol possess inhibitory effects on membrane bound Na^+-K^+ ATPase and 5′-nucleotidase (9,10). Replacement of part of the cholesterol of erythrocyte membrane ghost by cholest-4-en-3-one was accompanied by an inhibition of Na^+-K^+ATPase activity of the erythrocyte membrane (11). The inhibitory effect was mainly related to the depletion of membrane cholesterol and/or the incorporation of these oxidation derivatives into the cell membrane which can affect the fluidity of the plasma membrane (12,13).

The microsomal acyl-CoA desaturases are integral enzymes located in the endoplasmic reticulum where these enzymes control polyunsaturated fatty acids (PUFA) synthesis (14). Earlier studies have suggested that alterations in the physical state of microsomal membranes could be correlated with changes in activity of acyl-CoA desaturases (15,16). The effect of fluidity on the desaturating enzymes implies the existence of a self-regulatory mechanism in the membrane (17). The existence of high affinity OS binding sites in microsomes which are distinct from the cytosolic binding protein was also reported by Hwang (18).

OS have been found in the aortic tissues and plasma of animals fed atherogenic diets in a significantly higher level than the control animals (19,20). In human aortic tissues, the OS levels also were correlated with the severity of atherosclerosis (21,22). Because OS can inhibit or modify the membrane-bound enzymes involved in PUFA metabolism and because high-affinity binding sites for OS exist in microsomes, the present study was conducted to assess the effect of triol on linoleic acid metabolism and the fatty acid composition of porcine kidney cells (LLC-PK).

MATERIALS AND METHODS

Materials. [1-^{14}C]Linoleic acid (specific activity 55 mCi/mmol) and [1-^{14}C]oleic acid (specific activity 56 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin (BSA) fraction V and 5α-cholestane-3β,5,6β-triol (triol) were purchased from Sigma (St. Louis, MO). Fatty acids and their methyl esters

*To whom correspondence should be addressed at 205 Burnsides Research Laboratory, 1208 W. Pennsylvania Ave., Urbana, IL 61801.

Abbreviations: BCS, bovine calf serum; BSA, bovine serum albumin; CE, cholesteryl ester; FC, free cholesterol; FFA, free fatty acids; LLC-PK, porcine kidney; OS, oxysterols (oxidized derivatives of cholesterol); PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triglycerides; triol, 5α-cholestane-3β,5,6β-triol.

Copyright © 1995 by AOCS Press

and the thin-layer chromatographic reference standard mixtures, including phosphatidylcholine and the neutral lipids, were purchased from Nu-Chek-Prep (Elysian, MN). Coomassie blue G-250 based protein assay reagent was purchased from Pierce Co. (Rockford, IL). All other chemicals used in assays and tissue culture were purchased from Sigma. Solvents were reagent grade or high-performance liquid chromatography grade when required.

Cell culture. LLC-PK cells were purchased from American Type Culture Collection (ATCC; Rockville, MD), and stocks were frozen in liquid nitrogen. Cells were grown in M199 (Sigma) with 5% BCS (bovine calf serum, defined/supplemented) (HyClone Laboratories, Inc., Logan, UT) at 37°C in a 5% CO_2, humidified atmosphere. Cells were harvested using trypsin/EDTA (0.5 g trypsin, 0.2 g EDTA/L; Sigma). Medium containing triol was prepared by dissolving 0.5 g BSA (fraction V) (23) in 10 mL of M199, adjusting the pH to neutral, and adding 1 mg triol dissolved in 20 µL ethanol to make stock solution of 100 µg triol/mL. Then 90 mL of M199 were added to 10 mL of this stock solution to give 100 mL medium with 10 µg/mL of triol. The control medium was prepared similarly but with addition of BSA and ethanol without triol.

For an experiment, 60-mm diameter culture dishes were inoculated with 6.7×10^4 LLC-PK cells in 2 mL of medium. After 24 h of incubation, the medium was replaced with 2 mL of M199 with 5% BCS, antibiotic (100 units penicillin, 0.1 mg streptomycin, and 0.25 µg amphotericin B/mL, Sigma antibiotic antimycotic solution), and either 10 µg/mL triol or 0.02% ethanol for the control cells. After 24 h of incubation, the media were replaced with 2 mL of the appropriate media containing 100 nmol, 0.5 µCi/dish (50 µM) of [^{14}C]linoleate added as Na salt/BSA complex (1 µg fatty acid/11 µg BSA) (24).

In other experiments, the medium was enriched with 50 µM unlabeled linoleic acid as Na salt/BSA complex. After 1-, 6-, or 12-h incubations, the monolayers of the control and triol cells were washed gently with 2 mL of phosphate buffered saline, then harvested using trypsin/EDTA. The cells were collected in a test tube, and were pelleted by centrifugation. The supernatant was removed, 2 mL of methanol were added to the tube, the pellet was resuspended, and the tube was flushed with nitrogen.

Lipid analysis. The cellular lipids of the triol-treated and control cells were extracted according to the Folch method (25). The phospholipid fraction was separated from the neutral lipids on polysilicic acid gel impregnated glass fiber sheet (Gelman Science, Ann Arbor, MI) using as solvent system petroleum ether/diethyl ether/acetic acid (90:10:1, by vol). The phospholipids (PL) were transesterified using 14% BF_3 in methanol (Sigma) (26), and the fatty acid methyl esters were separated by gas–liquid chromatography as previously described (27). Butylated hydroxytoluene was added as antioxidant at a concentration of 0.005% (wt/vol) to all the solvents used for lipid extraction and chromatography. All operations were carried out under N_2.

The cellular fatty acid contents (expressed as µg/mg cell protein) were determined using heptadecanoic (17:0) acid as internal standard. The suspended cells in methanol were briefly sonicated (microprobe, output setting 60, 20 s, 4°C) in a Tekmar Sonic Disruptor (Model TM 50 T; Sonics and Materials Inc., Danbury, CT). Heptadecanoic acid was added to the methanolic cell suspension (10 µg in 10 µL $CHCl_3$). Two 100-µL aliquots were taken for protein determination using the microassay procedure following the instructions provided with the protein assay reagent kit (28), using albumin as standard. Cell lipids were then extracted, and the fatty acid methyl esters were prepared by methylation following saponification (29).

Fatty acids methyl esters were identified by comparing their relative retention times with those of authentic standards (Nu-Chek-Prep and Supelco, Bellefonte, PA). When 17:0 acid was used as internal standard, the fatty acid concentration as µg/mg cell protein was calculated relative to the internal standard peak (10 µg).

The cellular contents of PL, triol, and total as well as free cholesterol (FC) (as µg/mg cell protein) were determined as follows: The cells were incubated for 24 h with medium containing either triol (10 µg/mL) or ethanol for the control cells. The cells were collected, suspended in methanol, sonicated, and their protein content was determined as described above. One mL of this cell suspension in methanol was used to which 50 µg of 5α-cholestane (in 250 µL $CHCl_3$) was added as internal standard; then the cell lipid was extracted. The lipid residue was redissolved in exactly 10 mL $CHCl_3$, and two 1-mL aliquots were used for determining the total lipid phosphorus (30). The remaining lipid extract (8 mL) was dried under N_2 and derivatized to trimethylsilyl ether by addition of 500 µL of *N,N*-dimethylformamide (Fisher Scientific, Fair Lawn, NJ) and 500 µL of *bis*-trimethylsilyl-trifluoroacetamide (Supelco) and allowed to stand for 1 h at 60°C. The trimethylsilyl reagent was removed under a stream of nitrogen. The residue was redissolved in 50 µL isooctane, and 1–2 µL were analyzed by the gas-liquid chromatography as previously described (20). Quantitation of cholesterol and triol was accomplished by comparing their peak areas to that of the internal standard. The retention time of 5α-cholestane, cholesterol, and triol were 14.4, 18.34, and 21.87 min, respectively. For measuring the cellular total cholesterol content, the same procedure was followed but after saponification of the cell lipid with 2 M alcoholic KOH overnight at room temperature then extracting the unsaponifiable fraction, which was then silylated and injected into the gas chromatograph as described.

Conversion rate of linoleic acid to n-6 polyunsaturated acids. The cells were about 60% confluent when incubated in medium with or without triol for 24 h as described above. The medium was replaced with 2 mL of the same medium except that the fresh media contained 5% lipid-free BCS and 50 nmol/mL of [^{14}C]linoleic acid (100 nmol, 0.5 µCi/dish). After incubation at 37°C for 1, 6, or 12 h, the medium was removed. The cells were harvested, pelleted, and suspended in

methanol as described above. After sonication, two 100-μL aliquots were taken for protein determination (28), and another two 50-μL aliquots were used for measuring the total radioactivity. The total cellular uptake of linoleic acid as nmol/mg cell protein was calculated using the specific activity of linoleic acid (10,180 dpm/nmol). An additional 3.3 mL of methanol and 10 mL of $CHCl_3$ were added to the cell suspension, and lipids were extracted as described. Fatty acids from the extracted lipids were prepared by saponification with 2 M alcohol KOH and then methylation of free fatty acids (FFA) by 3 M methanolic HCl (Supelco) at 60°C for 1 h. The HCl/methanol was evaporated to dryness under N_2, and the methyl esters of linoleic, linolenic, arachidonic, and eicosapentaenoic acids as unlabeled carriers were added to each tube. The methyl esters were separated into dienes, trienes, tetraenes, and pentaenes, and the radioactivity in each band was measured as previously described (27). The percentage distribution of radioactivity in each band was determined, and the amounts (nmole) of labeled dienes, trienes, and tetraenes produced per mg cellular protein were calculated.

In order to measure the percentage distribution of the radioactivity between different lipid classes, the cell lipid was mixed with unlabeled carriers of standard PL and neutral lipids and separated into PL, FC, diglycerides, FFA, triglycerides (TG), and cholesteryl ester (CE) on silica gel G plates using the solvent system petroleum ether/diethyl ether/acetic acid (80:20:1, by vol). The bands were visualized by exposure to I_2 vapor, and the bands were each scraped from the plate and radioactivity was measured as described (27).

Statistical analysis. Results were expressed as means with their standard deviations. The statistical significance of difference between means was assessed by Student's *t*-test.

RESULTS

In this experiment we did not observe any significant change in the shape or viability of the cells when incubated with medium containing 10 μg triol/mL up to 24 h. The only difference was a slower growth rate of triol-treated cells as indicated by less cell protein as well as lower cell number/dish in the triol-treated cells (110 ± 5.4 μg protein/dish) than control cells (136 ± 4.1 μg protein/dish).

The fatty acid pattern of the cellular PL showed significant differences between the triol-treated and the control cells (Table 1). These differences were characterized by a substantial increase in linoleic acid (18:2n-6) and by decrease in arachidonic (20:4n-6) and docosatetraenoic (22:4n-6) acid percentages. Table 1 also shows that the ratios of total n-6 PUFA metabolites vs. total n-6 acids and 20:4n-6 vs. 18:2n-6 were significantly decreased in the triol-treated cells.

When the triol-treated and control cells were incubated with medium containing 50 μM [^{14}C]linoleic acid, the cellular uptake of this acid was significantly higher in the triol cells than in the control cells at 1-, 6-, and 12-h incubations (Fig. 1A). Table 2 shows that despite the increase of the pool size of linoleic acid in the triol-treated cells after 1- and 6-h incubations, a slight and nonsignificant increase was observed for its total n-6 PUFA metabolites (trienes + tetraenes) produced as nmol/mg cell protein in the triol-treated cells as compared to the control cells. This slight increase in total n-6 PUFA metabolites was the reflection of a significant increase in trienes produced as nmol/mg cell protein in the triol-treated cells. After 12-h incubation when the pool size of linoleic acid was increased to 318.9 nmol/mg cell protein in the triol-treated cells vs. 210.5 nmol/mg cell protein in the control cells, the n-6 PUFA metabolites (trienes + tetraenes), as well as tetraenes produced as nmol/mg cell protein, were increased in the triol cells (Table 2). The cellular protein contents of the control and triol-treated dishes were proportional to their cell counts in these dishes. In other words, the average protein content of the cells in the triol-treated dishes was about 19% less than the control dishes; the cell number in the triol-treated dishes was also about 20% less than the cell counts in the control dishes. Therefore, expressing our data of fatty acids or lipid contents per mg cellular protein or per cell number should give similar results.

When labeled dienes, trienes, and tetraenes produced by the triol or control cells, shown in Table 2 (as nmol/mg cell protein), were expressed as their relative percentages as shown in Figure 2, we found that the percentage of dienes was significantly increased while the percentages of tetraenes and total n-6 PUFA metabolites (trienes + tetraenes) were significantly decreased in the triol-treated cells. These changes are similar to those observed for the percentages of linoleic acid and its metabolites: 18:3n-6, 20:3n-6, 20:4n-6, and 22:4n-6 acids when the fatty acids of the cellular PL fraction of the triol and control cells were expressed as their relative percentages (Table 1).

TABLE 1
Fatty Acid Composition (mol%) of Phospholipids from Control and Triol Porcine Kidney Cells[a]

Fatty acid	Control cells (mol%)	Triol cells (mol%)	P^b
14:0	1.59 ± 0.14	2.66 ± 0.09	<0.01
16:0	14.12 ± 0.76	15.45 ± 0.47	NS[c]
16:1n-7	1.59 ± 0.21	1.99 ± 0.17	NS
16:1n-5	2.78 ± 0.23	2.37 ± 0.74	NS
18:0	8.90 ± 0.22	9.28 ± 1.65	NS
18:1n-9	19.15 ± 2.84	17.03 ± 1.83	NS
18:1n-7	3.92 ± 0.58	3.00 ± 0.12	NS
18:2n-6	22.06 ± 1.04	27.82 ± 0.73	<0.01
18:3n-6	2.08 ± 0.16	1.69 ± 0.37	NS
20:3n-6	2.16 ± 0.12	1.95 ± 0.21	NS
20:4n-6	17.63 ± 0.64	15.71 ± 0.11	<0.05
22:4n-6	3.20 ± 0.60	1.52 ± 0.20	<0.01
Total n-6 acids	47.13 ± 1.69	48.69 ± 0.46	NS
Total n-6 metabolites[d]	25.07 ± 0.67	20.85 ± 0.27	<0.02
n-6 Metabolites/n-6 acids	0.532 ± 0.005	0.428 ± 0.01	<0.001
20:4n-6/18:2n-6	0.799 ± 0.015	0.564 ± 0.018	<0.001

[a]Values are given as means ± standard deviation of three different incubations. Triol, 5α-cholestane-3β,5,6β-triol.
[b]Statistical analysis was by Student's *t*-test.
[c]NS, not significant.
[d]n-6 Metabolites are total n-6 acids other than 18:2n-6 acid.

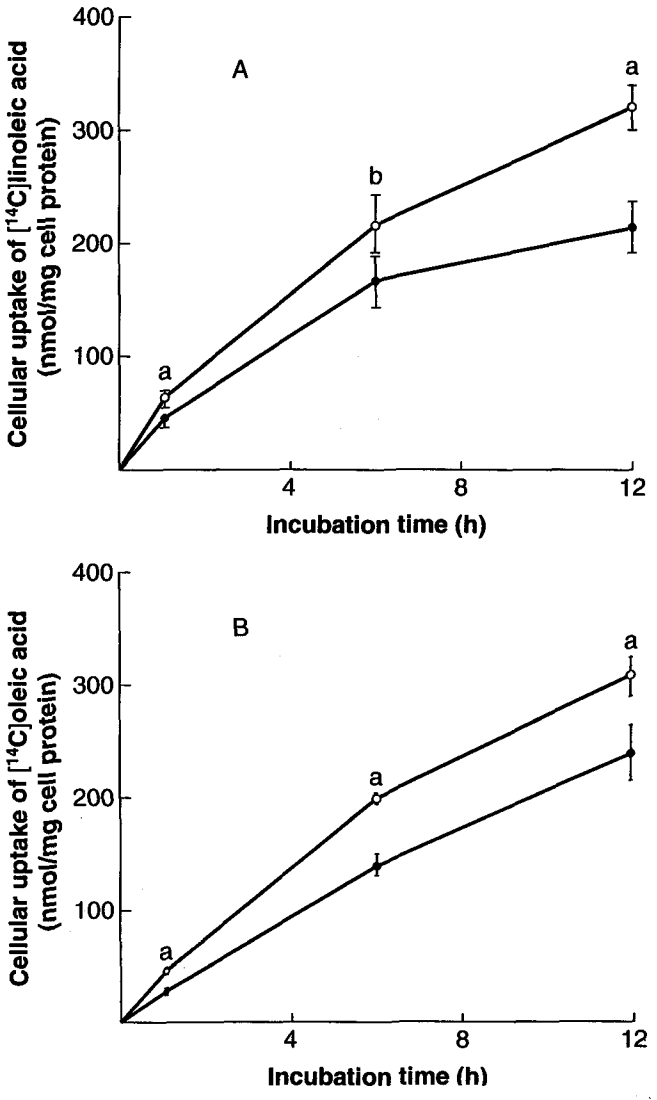


FIG. 1. A. Cellular linoleic acid uptake (as nmol/mg cell protein) by porcine kidney (LLC-PK) cells incubated in medium with (○) or without (●) cholestanetriol (10 μg/mL) for 24 h, then with medium containing 50 μM of [^{14}C]linoleic acid for 1, 6, and 12 h. a and b represent statistical differences at levels of $P < 0.001$ and $P < 0.02$, respectively, between control and triol cells. B. Cellular oleic acid uptake (as nmol/mg cell protein) by LLC-PK cells incubated in medium with (○) or without (●) cholestanetriol (10 μg/mL) for 24 h, then with medium containing 50 μM of [^{14}C]oleic acid for 1, 6, and 12 h. a Represents a statistical difference at a level of $P < 0.001$ as tested by Student's *t*-test.

Table 3 shows a significant increase in the cellular fatty acid concentration of palmitoleic (16:1n-7), stearic (18:0), and oleic (18:1n-9) acids (as μg/mg cell protein). The results in Table 3 also show significant increases in total n-6 acids, 18:2n-6, 20:3n-6, and 20:4n-6 concentrations, consistent with the results obtained in Table 2 when the triol and control cells were incubated with [^{14}C]linoleic acid for 12 h. A significant decrease of the ratios of total n-6 PUFA metabolites vs. total n-6 acids and of 20:4n-6 vs. 18:2n-6 also were observed. The cellular fatty acid contents in Table 3 also clarified that the increase of the trienes (as nmol/mg cell protein) observed in the [^{14}C]linoleic acid experiment (Table 2) was due to the accumulation of 20:3n-6 while 18:3n-6 was not increased from the levels of the control cells (Table 3).

Table 4 shows that the pool size of oleic acid in the triol-treated cells was significantly higher than in the control cells as a result of the increased uptake at 1-, 6-, and 12-h incubations (Fig. 1B). This increase in the pool size was accompanied by a significant increase of oleic acid (as nmol/mg cell protein) incorporated into both PL and TG of the triol-treated cells. No significant difference could be found between the number of nanomoles of oleic acid incorporated into FFA or CE of the triol-treated and control cells (Table 4). More than 85% of the total cellular oleic acid was incorporated into PL. The incorporation into TG did not exceed 10% of its pool size, indicating the selectivity of oleic acid esterification into PL.

Table 5 shows that when triol and control cells were incubated with [^{14}C]linoleic acid for 12 h, the pool size of the labeled fatty acids was significantly higher in the triol-treated cells. The labeled fatty acids incorporated into the PL and TG of the triol-treated cells also were significantly higher than in control cells and represented about 50 and 40% of the pool size of linoleic acid in both triol-treated and control cells, respectively. No significant difference was found between the number of nanomoles of labeled fatty acids incorporated into FFA or CE of triol and control cells (Table 5).

Since linoleic acid is metabolized to arachidonic acids in PK cells, we measured the numbers of nanomoles of dienes (18:2n-6) and tetraenes (20:4n-6 + 22:4n-6) incorporated into PL and TG per mg protein of the triol-treated and control cells. Table 6 shows that dienoic acids incorporated into either PL or TG were significantly higher in the triol-treated cells as compared to the control cells. Tetraenoic acids (mainly 20:4n-6) were slightly increased in the PL while significantly decreased in the TG of the triol-treated cells as compared to the control cells. These results agree with those previously reported (31) for arachidonic acid distribution under triol effect. In Table 5, the labeled fatty acids found in the FFA fraction in either triol or control cells did not exceed 0.5% of the pool size of linoleic acid. This indicates that the activation of linoleic acid to linoleoyl-CoA was not a rate-limiting step for linoleic acid conversion to arachidonic acid in either triol or control cells.

Our results in Table 7 show that triol decreased the levels of total as well as free cellular cholesterol (as μg/mg cell protein) in the triol-treated cells as compared to the control cells. It also shows that free triol was inserted into the cell membrane of the triol cells in a detectable level while it was absent from the control cells. Table 7 also shows that triol increased the cellular PL contents (as μg/mg cell protein).

DISCUSSION

The changes of the fatty acid percentage composition of the cellular PL in the triol-treated cells indicate that the conversion of linoleic acid to arachidonic acid was impaired in the

TABLE 2
[^{14}C]Linoleic Acid Metabolism in Control and Triol LLC-PK Cells[a]

	Incubation time[b]								
	1 h			6 h			12 h		
	Control cells	Triol cells	P[c]	Control cells	Triol cells	P	Control cells	Triol cells	P
Pool size of linoleic acid: 18:2n-6 (nmol/mg protein)	46.3 ± 6.0	60.8 ± 4.4	<0.001	165.3 ± 29.4	215.8 ± 25.9	<0.02	210.5 ± 22.1	318.9 ± 17.7	<0.001
Dienoic acids: 18:2n-6 (nmol/mg protein)	25.2 ± 4.5	36.7 ± 4.7	<0.001	68.9 ± 7.0	112.9 ± 20.2	<0.01	69.3 ± 3.7	139.4 ± 6.0	<0.001
Trienoic acids: 18:3n-6 + 20:3n-6 (nmol/mg protein)	6.8 ± 0.9	8.5 ± 0.6	<0.01	22.7 ± 5.6	28.4 ± 1.9	<0.05	23.1 ± 2.4	39.1 ± 1.4	<0.001
Tetraenoic acids: 20:4n-6 + 22:4n-6 (nmol/mg protein)	11.8 ± 1.6	12.3 ± 1.6	NS[d]	60.7 ± 14.0	61.2 ± 8.0	NS	99.0 ± 13.2	117.3 ± 10.0	<0.02
Total n-6 metabolites (nmol/mg protein)	18.6 ± 2.4	20.7 ± 2.0	NS	84.2 ± 17.7	89.6 ± 9.6	NS	122.1 ± 15.5	156.4 ± 11.0	<0.001
Tetraenes/dienes	0.48 ± 0.08	0.34 ± 0.06	<0.01	0.88 ± 0.14	0.55 ± 0.06	<0.001	1.43 ± 0.14	0.84 ± 0.05	<0.001
n-6 Metabolites/pool size of 18:2n-6	0.43 ± 0.04	0.36 ± 0.04	<0.02	0.55 ± 0.04	0.44 ± 0.03	<0.001	0.64 ± 0.02	0.53 ± 0.02	<0.001

[a]Values are given as means ± standard deviation of eight incubations from two different experiments. Abbreviation as in Table 1.
[b]Porcine kidney (LLC-PK) cells were incubated in medium with (triol) or without (control) cholestanetriol (10 µg/mL) for 24 h, then with medium containing 50 µM [14C]linoleic acid for 1, 6, or 12 h.
[c]Statistical analysis was by Student's *t*-test.
[d]NS, not significant.

triol cells as evidenced by the decrease of the ratios total n-6 metabolites vs. total n-6 acids and 20:4n-6 vs. 18:2n-6 (32).

After 1- and 6-h incubations with [^{14}C]linoleic acid, despite the increase of linoleic acid pool size in the triol-treated cells, neither total n-6 metabolites nor arachidonic acid accumulation was increased while trienes did accumulate in the triol cells. The increased accumulation of labeled trienes in the triol-treated cells could be an indication of decreased Δ5 desaturase activity in the triol-treated cells. The increased accumulation of trienes in triol-treated cells was due to the accumulation of 20:3n-6 while 18:3n-6 was within the levels of the control cells. This rules out the possible inhibition of elongase enzyme by triol since no accumulation of 18:3n-6, the substrate for the elongase enzyme, was observed in the triol-treated cells. After 12-h incubation when the linoleic acid pool size in the triol-treated cells was increased 50%, dienes (18:2n-6) were increased 100%, trienes increased 65%, while arachidonic acid was increased only 18% as compared to the control cells.

Any change of arachidonic acid utilization for prostacyclin synthesis may alter its level under triol effect. We must mention here that LLC-PK cell line used in the present study lacks cyclooxygenase enzyme and does not produce prostaglandin. Triol also does not induce its effect through free radicals (33) which excludes any possible change of arachidonic acid due to oxidative alteration under triol effect.

Although linoleic acid uptake was increased in the triol-treated cells, the fatty acid percentage composition of cellular PL did not show any difference in the relative percentage of total n-6 acids between triol and control cells. Analysis of the fatty acids of total cellular lipids after 12-h incubation with 50 µM linoleic acid showed that the fatty acid concentrations (as µg/mg cell protein) of palmitoleic, stearic, and oleic acids were increased as well as total n-6 acids were increased in the triol cells. Indeed Peng *et al.* (34) found that triol was among the OS which enhanced the *de novo* synthesis of fatty acids by 42% in cultured cells. Palmitic acid (16:0) synthesized by the cells can be chain-elongated to stearic (18:0) then desaturased by Δ9 desaturase to oleic acid (18:1n-9).

The increased uptake of fatty acids by the triol-treated cells could be related to changes of the membrane-associated functions and properties (35). Proteins that have important cellular functions such as transporters, receptors, and enzymes are embedded in cholesterol–PL bilayer of the cell membrane. Any change of the lipid content of the bilayer may have a modulatory influence on the activities of these membrane proteins (36). The increase uptake of fatty acids might involve activation of an uptake mechanism caused by triol-induced changes in the plasma membrane (31).

The increased cellular uptake of oleic and linoleic acids in triol-treated cells affected their distributions between cellular lipids. In the present study, oleic acid incorporation was significantly increased into PL and TG of the triol-treated cells with a great selectivity of incorporation into PL, as previously observed by Seillan (31). Our results, however, are not in full agreement with those obtained by Seillan (31), who found that the percentage of oleic acid incorporated into PL was increased while in TG was decreased in the triol-treated cells. This discrepancy could be explained by the increased pool size of oleic acid in our study which makes oleic acid available for incorporation into both PL and TG fractions. In the study by Seillan (31), cellular uptake did not change; there-

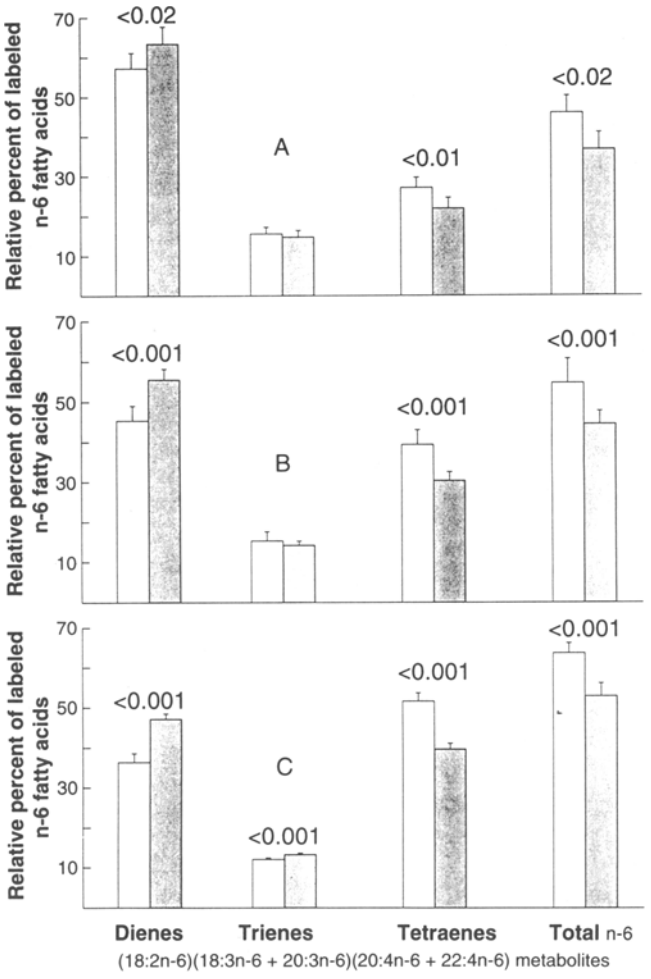


FIG. 2. Percent distribution of labeled dienes, trienes, and tetraenes and total n-6 metabolites (trienes + tetraenes) in porcine kidney cells incubated in medium with (■) or without (□) cholestanetriol (10 μg/mL) for 24 h, then with medium containing 5% lipid free bovine calf serum and 50 μM of [^{14}C]linoleic acid for one (A), six (B), or twelve (C) hours.

fore, oleic acid may be preferably incorporated into PL at the expense of oleic acid incorporation into TG. We used triol at concentration of 10 μg/mL medium while the maximum concentration Seillan (31) used was 4 μg/mL (31). When we used triol up to 5 μg/mL, we did not get any significant change in the cellular uptake of linoleic acid (data are not shown). This difference also may be attributed to the different sensitivity to triol between the artery cells used by Seillan (31) and the PK cells used in our study. It also indicates that triol is less cytotoxic to PK cells at the same level where it is highly toxic to the arterial cells. In our experiment we did not observe any significant change in the shape or viability of the cells when incubated with medium containing 10 μg triol/mL up to 24 h. The only difference was a slower growth rate of triol-treated cells as indicated by the less cell protein as well as cell number/dish in the triol cells than the control cells.

The decrease of total as well as FC in the triol-treated cells may have resulted from the inhibition of cholesterol synthesis in these cells (37). The decrease of the free cellular cholesterol and the increase of PL contents in the triol-treated

TABLE 3
Fatty Acid Contents (as μg/mg cell protein) of Total Lipids from Control and Triol LLC-PK Cells[a]

Fatty acid	Control cells (μg/mg cell protein)	Triol cells (μg/mg cell protein)	P^b
16:0	58.18 ± 4.43	54.34 ± 1.25	NS[c]
16:1n-7	4.43 ± 0.25	7.51 ± 0.43	<0.001
16:1n-5	6.62 ± 1.11	8.14 ± 0.12	NS
18:0	75.62 ± 8.49	117.01 ± 3.50	<0.01
18:1n-9	95.90 ± 6.90	125.52 ± 5.29	<0.01
18:1n-7	25.09 ± 0.91	29.65 ± 4.34	NS
18:2n-6	38.20 ± 2.05	85.31 ± 2.05	<0.001
18:3n-6	4.11 ± 0.60	5.29 ± 0.52	NS
20:3n-6	7.26 ± 0.68	13.06 ± 1.56	<0.02
20:4n-6	69.53 ± 5.20	108.69 ± 6.99	<0.01
22:4n-6	19.09 ± 1.50	20.45 ± 1.59	NS
Total n-6 acids	135.77 ± 8.72	232.81 ± 10.96	<0.001
Total n-6 metabolites[d]	97.57 ± 6.99	147.50 ± 9.76	<0.01
n-6 Metabolites/ n-6 acids	0.72 ± 0.01	0.63 ± 0.01	<0.001
20:4n-6/18:2n-6	1.82 ± 0.04	1.27 ± 0.07	<0.001

[a]Values are given as means ± standard of three different experiments. See Tables 1 and 2 for abbreviations.
[b]Statistical significant differences were calculated by Student's *t*-test.
[c]NS, not significant.
[d]n-6 Metabolites are total n-6 acids other than 18:2n-6 acid.

cells would result in a significant decrease in the cholesterol/PL ratio. The insertion of triol into the cellular membranes also can modify membrane fluidity (13) and consequently membrane-bound enzymes (10,11). OS have been found to strongly decrease the membrane fluidity of the cell membrane (38), as well as the internal cell membranes and lipid-containing organelles (13). A strong correlation also was found between their inhibitory effect on 3 hydroxy-3-methylglutaryl–CoA reductase and their decreasing effect on the membrane fluidity (39).

Cholesterol feeding which increases the level of oxysterols *in vivo* (19,20) has been shown to reduce the arachidonic acid percentage and the ratio 20:4n-6 vs. 18:2n-6 in plasma and liver lipids (40,41). Our results are in agreement with those reported by Kou *et al.* (42) who found that the percentage of linoleic acid was increased while arachidonic acid was decreased in the PL fractions of the platelets incubated with 25-hydroxycholesterol. If similar changes occur in aortic cells by triol or other oxysterols, one can speculate that our results may explain the elevation of linoleic acid and the decrease of arachidonic and docosatetraenoic (22:4n-6) acids percentages in the PL of the coronary arteries of cardiac sudden death patients as compared to the controls (43), considering that OS have been found in relatively higher levels in plasma (19,44) and aortic tissues (19) of atherosclerotic animals. These changes were related to the impaired metabolism or accumulation of linoleic acid in the coronary artery wall in cases of sudden cardiac death (43).

In conclusion, the present study showed that essential fatty acid metabolism is perturbed along with enhanced uptake of

TABLE 4
Distribution of Cellular [^{14}C]Oleic Acid Between the Lipid Classes of Control and Triol LLC-PK Cells[a]

	Incubation time[b]								
	1 h			6 h			12 h		
	Control cells	Triol cells	P[c]	Control cells	Triol cells	P	Control cells	Triol cells	P
Total pool size of oleic acid: 18:1n-9 (nol/mg cell protein)	29.6 ± 4.4	47.1 ± 2.9	<0.001	139.1 ± 10.6	198.9 ± 6.1	<0.001	238.8 ± 27.2	307.8 ± 31.3	<0.001
Phospholipid (nmol/mg cell protein)	25.7 ± 4.1	39.9 ± 2.8	<0.01	128.0 ± 9.9	175.9 ± 7.4	<0.001	214.9 ± 18.2	267.8 ± 19.2	<0.01
Triglycerides (nmol/mg cell protein)	1.25 ± 0.72	3.82 ± 0.87	<0.01	4.5 ± 1.4	15.9 ± 2.2	<0.001	14.4 ± 8.5	32.7 ± 11.4	<0.05
Free fatty acids (nmol/mg cell protein)	0.74 ± 0.17	0.60 ± 0.07	NS[d]	0.79 ± 0.16	1.02 ± 0.13	NS	1.0 ± 0.5	1.44 ± 0.04	NS
Cholesterol ester (nmol/mg cell protein)	0.17 ± 0.05	0.15 ± 0.02	NS	0.18 ± 0.02	0.56 ± 0.15	<0.01	0.33 ± 0.18	0.58 ± 0.26	NS

[a]Values are given as means ± standard deviation of four different experiments. See Tables 1 and 2 for abbreviations.
[b]LLC-PK cells were incubated in medium with (triol) or without (control) cholestanetriol (10 μg/mL) for 24 h, then with medium containing 50 μM of [^{14}C]oleic acid for 1, 6, or 12 h.
[c]Statistical analysis was by Student's *t*-test.
[d]NS, not significant.

TABLE 5
Distribution of Cellular Labeled n-6 Fatty Acids Between Lipid Classes of Control and Triol LLC-PK Cells[a]

Treatment[b]	The pool size of 18:2n-6 (nmol/mg cell protein)	Phospholipid (nmol/mg cell protein)	Triglycerides (nmol/mg cell protein)	Free fatty acid (nmol/mg cell protein)	Cholesterol ester (nmol/mg cell protein)
Control cells	197.53 ± 9.90	110.57 ± 6.60 (56.0)	78.74 ± 2.93 (40.0)	0.93 ± 0.38 (0.47)	1.59 ± 0.58 (0.80)
Triol cells	235.80 ± 15.90	126.52 ± 8.20 (53.65)	102.05 ± 8.02 (43.3)	1.14 ± 0.3 (0.48)	1.81 ± 0.51 (0.77)
P[c]	<0.001	<0.01	<0.001	NS[d]	NS

[a]Values are given as means ± standard deviation of four different experiments. Values in parentheses represent the percent of pool size of linoleic acid. See Tables 1 and 2 for abbreviations.
[b]LLC-PK cells were incubated in medium with (triol) or without (control) cholestanetriol (10 μg/mL) for 24 h, then in medium containing 50 μM [14]linoleic acid for 12 h. The cellular lipid was extracted, and the radioactivity distribution between lipid classes was measured as described in the text.
[c]Statistical analysis was by Student's *t*-test.
[d]NS, not significant.

TABLE 6
Distribution of Cellular Labeled n-6 Dienoic and Tetraenoic Acids Between Triglycerides and Phospholipids of Control and Triol LLC-PK Cells[a]

	Phospholipid		Triglycerides	
Treatment[b]	Dienoic acids (nmol/mg cell protein)	Tetraenoic acids (nmol/mg cell protein)	Dienoic acids (nmol/mg cell protein)	Tetraenoic acids (nmol/mg cell protein)
Control cells	54.29 ± 5.13	22.78 ± 2.52	40.57 ± 3.04	22.26 ± 1.48
Triol cells	71.65 ± 7.73	25.44 ± 4.81	65.11 ± 5.66	18.53 ± 1.83
P[c]	<0.001	NS[d]	<0.001	<0.01

[a]Values are given as means ± standard deviation of four different experiments. See Tables 1 and 2 for abbreviations.
[b]LLC-PK cells were incubated in medium with (triol) or without (control) cholestanetriol (10 μg/mL) for 24 h, then with medium containing 50 μM [^{14}C]linoleic acid for 12 h. The cellular lipid was extracted, and the total radioactivity as well as the radioactivity distribution between phospholipids (PL) and triglycerides (TG) fractions were measured using a small portion of the lipid extract. In the remaining portion of the lipid extract, the radioactivity distribution between dienoic and tetraenoic acids of the TG and PL fractions was determined as described under the Materials and Methods section.
[c]Statistical analysis was by Student's *t*-test.
[d]NS, not significant.

TABLE 7
Total and Free Cholesterol and Phospholipid Contents in LLC-PK Cells Incubated in Medium with (triol) or without (control) Cholestanetriol (10 μg/mL) for 24 h[a]

	Control cells	Triol cells	p^b
Total cholesterol (μg/mg cell protein)	51.79 ± 4.24	39.86 ± 1.39	<0.001
Free cholesterol (μg/mg cell protein)	41.59 ± 3.42	29.90 ± 1.85	<0.001
Triol (μg/mg cell protein)	ND[c]	15.50 ± 3.07	
Total lipid phosphorus (μg/mg cell protein)	26.64 ± 0.81	34.71 ± 1.86	<0.01

[a]Values are given as means ± standard deviation of six different incubations. See Tables 1 and 2 for abbreviations.
[b]Statistical significant differences were calculated by Student's *t*-test.
[c]Not detectable.

linoleic acid in the triol cells. These changes disturbed the ratio between linoleic acid and its physiologically active metabolite arachidonic acid within the cellular membrane PL and affected their distribution between the cellular lipids.

ACKNOWLEDGMENT

This research was funded by the Wallace Genetic Foundation (Rye Brook, NY).

REFERENCES

1. Smith, L.L., and Johnson, B.H. (1989) Biological Activities of Oxysterols, *Free Rad. Biol. Med. 7*, 285-332.
2. Peng, S.K., Taylor, C.B., Mosbach, E.H., Huang, W.Y., Hill, J., and Mikkelson, B. (1982) Distribution of 25-Hydroxy-cholesterol in Plasma Lipoproteins and Its Role in Atherogenesis, *Atherosclerosis 41*, 395–402.
3. Kandutsch, A.A., and Chen, H.W. (1977) Consequences of Blocked Sterol Synthesis in Cultured Cells. DNA Synthesis and Membrane Composition, *J. Biol. Chem. 252*, 409–415.
4. Peng, S.K., Taylor, C.B., Tham, P., Werthessen, N.T., and Mikkelson, B. (1978) Effect of Auto-oxidation Products from Cholesterol on Aortic Smooth Muscle Cells, *Arch. Pathol. Lab. Med. 102*, 57–61.
5. Kandutsch, A.A., Chen, H.W., and Heiiger, H.J. (1978) Biological Activity of Some Oxygenated Sterols, *Science 201*, 498–501.
6. Smith, L. (1987) Cholesterol Autoxidation 1981–1986, *Chem. Phys. Lipids 44*, 87–125.
7. Sander, B.D., Addiss, P.B., Park, S.W., and Smith, D.E. (1989) Quantitation of Cholesterol Oxidation Products in a Variety of Foods, *J. Food Protection 52*, 109–114.
8. Bascoul, J., Domergue, N., Olle, M., and Crastes de Paulet, A. (1986) Autoxidation of Cholesterol in Tallows Heated Under Deep Frying Conditions: Evaluation of Oxysterols by GLC and TLC–FID, *Lipids 21*, 383–388.
9. Peng, S.K., Hill, J.C., Morin, R.J., and Taylor, C.B. (1985) Influence of Cholesterol Oxidation Derivative on Membrane Bound Enzymes in Cultured Aortic Smooth Muscle Cells, *Proc. Soc. Exp. Biol. Med. 180*, 126–132.
10. Peng, S.K., and Morin, R.J. (1987) Effects on Membrane Function by Cholesterol Oxidation Derivatives in Cultured Aortic Smooth Muscle Cells, *Artery 14*, 85–99.
11. Seiler, D., and Fiehn, W. (1976) Effect of Cholesterol Oxidation on (Na^+, K^+) ATPase Activity of Erythrocyte Membranes, *Experientia 32*, 849–850.
12. Epand, R.M., and Bottega, R. (1987) Modulation of the Phase Transition Behaviour of Phosphatidylethanolamine by Cholesterol and Oxysterols, *Biochemistry 26*, 1820–1825.
13. Bruneau, C., Staedel-Flaig, C., Cremel, G., Leroy, C., Beck, J.P., and Hubert, P. (1987) Influence of Lipid Environment on Insulin Binding in Cultured Hepatoma Cells, *Biochim. Biophys. Acta 928*, 287–296.
14. Brenner, R.R. (1984) Effect of Unsaturated Acids on Membrane Structure and Enzyme Kinetics, *Prog. Lipid Res. 23*, 69–96.
15. Christon, R., Fernandez, Y., Cambon-Gros, C., Periquet, A., Deltour, P., Leger, C., and Mitjavila, S. (1988) The Effect of Dietary Essential Fatty Acid Deficiency on the Composition and Properties of the Liver Microsomal Membrane of Rats, *J. Nutr. 118*, 1311–1318.
16. Garda, H.A., and Brenner, R.R. (1984) Short-chain Aliphatic Alcohols Increase Rat-Liver Microsomal Membrane Fluidity and Affect the Activities of Some Microsomal Membrane-Bound Enzymes, *Biochim. Biophys. Acta 769*, 160–170.
17. Brenner, R.R. Castuma, C.E., and Garda, H.A. (1986) Possible Mechanisms by Which Microsomal Lipid Bilayer Composition Modify Bound Enzyme Kinetics, *Prog. Lipid Res. 25*, 47–52.
18. Hwang, P.L.H. (1990) High Affinity Binding Sites for Oxygenated Sterols in Rat Liver Microsomes: Possible Identity with Antiestrogen Binding Sites, *Biochim. Biophys. Acta 1033*, 154–161.
19. Hodis, H.N., Crawford, D.W., and Sevanian, A. (1991) Cholesterol Feeding Increases Plasma and Aortic Tissue Cholesterol Oxide Levels in Parallel: Evidence for the Role of Cholesterol Oxidation in Atherosclerosis, *Atherosclerosis 89*, 117–126.
20. Mahfouz, M., Zhou, Q., and Kummerow, F.A. (1994) Cholesterol Oxides in Plasma and Lipoproteins of Magnesium-Deficient Rabbits and Effects of Their Lipoproteins on Endothelial Barrier Function, *Magnesium Research 7*, 207–222.
21. Brooks, C.J., Harland, W.A., and Steel, G. (1966) Squalene, 26-Hydroxycholesterol and 7-Ketocholesterol in Human Atheromatous Plaques, *Biochim. Biophys. Acta 125*, 620–622.
22. Smith, L.L., and Van Lier, J.E. (1970) Sterol Metabolism. Part 9. 26-Hydroxycholesterol Levels in the Human Aorta, *Atherosclerosis 12*, 1–14.
23. Boissonneault, G., and Heiniger, H.J. (1984) 25-Hydroxycholesterol-induced Elevation in ^{45}Ca Uptake: Correlation with Depressed DNA Synthesis, *J. Cell Physiol. 120*, 151–156.
24. Mahfouz, M., Johnson, S., and Holman, R.T. (1980) The Effect of Isomeric *Trans*-18:1 Acids on the Desaturation of Palmitic, Linoleic and Eicosa-8, 11, 14-Trienoic Acids by Rat Liver Microsomes, *Lipids 15*, 100–107.
25. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
26. Morrison, W.R., and Smith, L.M. (1964) Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride-Methanol, *J. Lipid Res. 5*, 600–608.
27. Mahfouz, M., Smith, T.L., and Kummerow, F.A. (1989) Changes of Linoleic Acid Metabolism and Cellular Phospholipid Fatty Acid Composition in LLC-PK Cells Cultured at Low Magnesium Concentrations, *Biochim. Biophys. Acta 1006*, 70–74.
28. Marcat, M., and Gerbant, L. (1982) An Improvement of the Comassie Blue Dye Binding Method Allowing an Equal Intensity to Various Proteins: Application to Cerebrospinal Fluid, *Clin. Chim. Acta 122*, 93–101.
29. DeSchrijver, R., and Privett, O.S. (1982) Interrelationship Between Dietary *Trans* Fatty Acids and the 6- and 9-Desaturases in the Rat, *Lipids 17*, 27–34.

30. Eng, L.F., and Noble, E.P. (1968) The Maturation of Rat Brain Myelin, *Lipids 3*, 157–162.
31. Seillan, C. (1990) Oxysterol Mediated Changes in Fatty Acid Distribution and Lipid Synthesis in Cultured Bovine Aortic Smooth Muscle Cells, *Lipids 25*, 172–176.
32. Cunnane, S.C., and Wahle, K.W. (1981) Zinc Deficiency Increases the Rate of Δ6 Desaturation of Linoleic Acid in Rat Mammary Tissue, *Lipids 16*, 771–774.
33. Hennig, B., Boissonneault, G.A., Fiscus, L.J., and Marra, M.E. (1988) Effect of Vitamin E on Oxysterol- and Fatty Acid Hydroperoxide-induced Changes of Repair and Permeability Properties of Cultured Endothelial Cell Monolayers, *Int. J. Vit. Nutr. Res. 58*, 41–47.
34. Peng, S.K., Morin, R.J., Tham, P., and Taylor, B. (1985) Effects of Oxygenated Derivatives of Cholesterol on Cholesterol Uptake by Cultured Aortic Smooth Muscle Cells, *Artery 13*, 144–164.
35. Lichtenstein, A., and Brecher, P. (1983) Esterification of Cholesterol and 25-Hydroxycholesterol by Rat Liver Microsomes, *Biochim. Biophys. Acta 751*, 340–348.
36. Spector, A.A., and Yorek, M.A. (1985) Membrane Lipid Composition and Cellular Function, *J. Lipid Res. 26*, 1015–1035.
37. Taylor, F.R., Saucier, S.E., Shown, E.P., Parish, E.J., and Kandutsch, A.A. (1984) Correlation Between Oxysterol Binding to a Cytosolic Binding Protein and Potency in the Repression of Hydroxymethylglutaryl Coenzyme A Reductase, *J. Biol. Chem. 259*, 12382–12387.
38. Rooney, M.W., Yachnin, S., Kucuk, O., Lis, L.J., and Kaufman, J.W. (1985) Oxygenated Cholesterols Synergistically Immobilize Acyl Chains and Enhance Protein Helical Structure in Human Erythrocyte Membranes, *Biochim. Biophys. Acta 820*, 33–39.
39. Reichert, L., Castagna, M., Beck, J.P., Rong, S., Luu, B., and Ourisson, G. (1984) Growth-rate-related and Hydroxysterol-induced Changes in Membrane Fluidity of Cultured Hepatoma Cells: Correlation with 3-Hydroxy-3-methyl glutaryl CoA Reductase Activity, *Biochem. Biophys. Res. Commu. 120*, 192–198.
40. Huang, Y.S., and Horrobin, D.F. (1987) Effect of Dietary Cholesterol and Polyunsaturated Fats on Plasma and Liver Lipids in Guinea Pigs, *Ann. Nutr. Metab. 31*, 18–28.
41. Huang, Y.S., Manku, M.S., and Horrobin, D.F. (1984) The Effects of Dietary Cholesterol on Blood and Liver Polyunsaturated Fatty Acids and on Plasma Cholesterol in Rats Fed Various Types of Fatty Acid Diet, *Lipids 19*, 664–672.
42. Kou, I.L., Pikul, J., and Kummerow, F.A. (1991) Influence of 26-Hydroxycholesterol on the Composition and Function of Gel-filtered Platelets, *J. Am. Coll. Nutr. 10*, 114–122.
43. Loustarinen, R., Boberg, M., and Saldeen, T. (1993) Fatty Acid Composition in Total Phospholipids of Human Coronary Arteries in Sudden Cardiac Death, *Atherosclerosis 99*, 187–193.
44. Stalenhoef, A.F.H., Kleinveld, H.A., Kosmeijer, S., Demacker, P.N.M., and Katan, M.B. (1993) *In Vivo* Oxidized Cholesterol in Atherosclerosis, *Atherosclerosis 98*, 113–114.

[Received February 24, 1994, and in final revised form September 26, 1995; Revision accepted September 27, 1995]

Effects of Non-β-Oxidizable Sulfur-Substituted Fatty Acid Analogues on Synthesis and Secretion of Triacylglycerol and Cholesterol in Cultured Rat Hepatocytes

Jon Skorve[a,*], Arild C. Rustan[b] and Rolf K. Berge[a]

[a]Department of Clinical Biology, Division of Biochemistry, University of Bergen, Haukeland University Hospital, 5021 Bergen, Norway and [b]Department of Pharmacology, Institute of Pharmacy, University of Oslo, 0316 Oslo, Norway

ABSTRACT: The mechanisms behind the hypolipidemic effect of two sulfur-substituted fatty acid analogues, 3-thiadicarboxylic acid and tetradecylthioacetic acid, have been investigated in cultured hepatocytes. There was a dose-dependent reduction in incorporation of [^{3}H]water into triacylglycerol and diacylglycerol when tetradecylthioacetic acid was added to rat hepatocytes cultured in the presence of 200 μM oleic acid. Tetradecylthioacetic acid also increased the oxidation of [^{14}C]palmitic acid compared to oleic acid, inhibited the incorporation of radiolabeled precursors into diacylglycerol to a greater extent than into triacylglycerol, and reduced the secretion of triacylglycerol more than its synthesis. A stimulation, rather than a reduction, in glycerolipid synthesis and secretion by tetradecylthioacetic acid was observed when oleic acid was omitted from the culture medium. When 3-thiadicarboxylic acid was added to cultured hepatocytes, the effects on glycerolipid synthesis were generally similar to those observed with tetradecylthioacetic acid, but 3-thiadicarboxylic acid did not increase the oxidation of [^{14}C]palmitic acid. The two fatty acid analogues also had different effects on the synthesis and secretion of cholesterol and cholesteryl esters—3-thiadicarboxylic acid reduced the incorporation of [^{3}H]water into synthesized and secreted cholesterol and cholesteryl esters, whereas tetradecylthioacetic acid only reduced the secretion of cholesteryl esters without affecting its synthesis. It is concluded that tetradecylthioacetic acid increases the oxidation of fatty acids and reduces the synthesis and secretion of glycerolipids. 3-Thiadicarboxylic acid reduces the synthesis and secretion of both glycerolipids and cholesterol to approximately the same extent without a concomitant increase in the oxidation of fatty acids.
Lipids 30, 987–994 (1995).

Sulfur-substituted dicarboxylic and monocarboxylic fatty acid analogues can reduce the level of plasma triacylglycerol and cholesterol (1–3), but the mechanism is not fully understood. Feeding 3-thiadicarboxylic acid [HOOC-CH_2-S-$(CH_2)_{10}$-S-CH_2-COOH] to rats decreased plasma levels of free fatty acids, very low density lipoproteins triacylglycerol, low density lipoproteins triacylglycerol, and low density lipoprotein cholesterol levels. However, the composition of the various plasma lipoprotein fractions was essentially unchanged (4).

In the liver, 3-thiadicarboxylic acid increased the oxidation of of fatty acids while decreasing the level of triacylglycerol, and it also reduced the activities of several key enzymes in lipid metabolism, including phosphatidate phosphohydrolase and 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase (3–5). 3-Thiadicarboxylic acid is also a potent peroxisome proliferator in rodents, increasing peroxisomal ß-oxidation up to tenfold (1).

The monocarboxylic acid, tetradecylthioacetic acid [CH_3-$(CH_2)_{13}$-S-CH_2-COOH] is structurally distinct from 3-thiadicarboxylic acid. This analogue has properties closely resembling normal fatty acids, except that it is unable to undergo β-oxidation (6). It is activated to its CoA-ester *in vitro* more readily than the dicarboxylic acid (7). 3-Thiadicarboxylic acid is far more polar than the monocarboxylic acid, and does not seem to be incorporated into glycerolipids. In contrast, tetradecylthioacetic acid is incorporated into triacylglycerols and phospholipids in rat liver (8), in isolated rat hepatocytes (9), and in hepatoma cells (10). In rat liver *in vivo*, most of the administered tetradecylthioacetic acid was found in the phospholipid fraction (11).

Despite structural differences, tetradecylthioacetic acid and 3-thiadicarboxylic acid have nearly the same hypolipidemic effects, but tetradecylthioacetic acid is less potent in decreasing the hepatic level of triacylglycerol and in inducing peroxisomal proliferation (1,2). The mode of action of these fatty acid analogues *in vivo* is presumed to involve decreased availability of fatty acids due to increased β-oxidation, as well as changes in the activities of key enzymes involved in triacylglycerol and cholesterol biosynthesis (4,5).

Primary cultures of rat hepatocytes are a well-characterized system for studying synthesis and secretion of lipoproteins and bile acids (12–14). We have, therefore, utilized these cells to study the *in vitro* effects of the non-β-oxidizable sulfur-substituted fatty acid analogues 3-thiadicarboxylic acid and tetradecylthioacetic acid on lipid metabolism. We have examined the incorporation of various labeled precursors into

*To whom correspondence should be addressed.

Abbreviations: Apo, apolipoprotein; DME, Dulbecco's modified Eagle's medium; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

synthesized and secreted lipids, as well as the oxidation of fatty acids in cultured hepatocytes.

MATERIALS AND METHODS

Chemicals. Arginine-free Dulbecco's modified Eagle's medium (DME) and gentamicin (10 mg/mL) were obtained from Flow Lab. (Irvine, Ayrshire, United Kingdom). The medium was supplemented with ornithine (0.5 mM), HEPES (20 mM), and $NaHCO_3$ (24 mM) from Sigma Chemical Co. (St. Louis, MO). [1,2,3-^{3}H]glycerol (200 Ci/mol), [^{3}H]water (18 Ci/mol), [1-^{14}C]palmitic acid (56 Ci/mol), and [1-^{14}C]oleic acid (58 Ci/mol) were obtained from New England Nuclear (Dreieich, Germany). Collagenase (type 1), bovine insulin, oleic acid, and bovine serum albumin (essentially fatty acid-free) were delivered by Sigma. Ultroser G (a serum substitute) was delivered by Gibco, Life Technologies Inc. (Gaithersburg, MD). Tissue culture dishes/flasks were supplied by Costar (Cambridge, MA). Thin-layer chromatography (TLC) plates (Silicagel F 1500) were purchased from Schleicher & Schuell (Dassel, Germany). All other chemicals and solvents were high-purity commercial materials.

Sulfur-substituted fatty acid analogus (3-thia fatty acids). Stock solutions (10 mM) of 3-thiadicarboxylic acid and tetradecylthioacetic acid were prepared as described earlier (15,16). The 3-thia fatty acids were dissolved in 0.1 mM NaOH at 50–60°C before addition to the culture medium.

Preparation of cultured hepatocytes. Hepatocytes were prepared according to Seglen (17), with some modifications (14). In short, the rats were anesthetized with barbiturate, and hepatocytes were isolated by collagenase perfusion, washed and plated in DME containing 20 mM HEPES, Ultroser G (2%), and gentamicin (50 μg/mL) at a density of 3×10^6 cells/dish or culture flask (1×10^6/mL). The culture medium was replaced after 6–7 h (2 mL/dish, identical medium as before). The cells were incubated at 37°C in air/CO_2 (19:1).

Measurement of cell-associated and secreted lipids. After overnight incubation (18 h), the culture medium was removed and the cells were rinsed twice with serum-free DME medium to remove unattached and damaged cells. The cells were then changed to DME containing glucose (25 mM), HEPES (20 mM), gentamicin (50 μg/mL), and the various isotopes and compounds indicated later and in legends to the figures and in the tables.

Incorporation of tritiated water. Two mL incubation medium with [^{3}H]water (3 mCi/mL), with or without oleic acid/bovine serum albumin (200 μM/80 μM) was added to hepatocytes cultured in plastic flasks (25 cm^2). Cells without oleic acid were incubated with 80 μM bovine serum albumin alone. The respective thia-fatty acids (from heated stock solutions) were then added to the culture medium, and the cells were incubated for periods up to 9 h.

Incorporation of glycerol. Two mL incubation medium (as described previously), containing [1,2,3-^{3}H]glycerol (5 μCi/ml, 25 μM), with or without oleic acid/bovine serum albumin (200 μM/80 μM), and the respective thia fatty acids were added to the cells. The cells were incubated for periods up to 1 h. In pulse-chase experiments, the cells were preincubated for 1 h in medium with oleic acid and 5 μCi/mL [^{3}H]glycerol (25 μM), as described previously. The medium was then changed and the cells chased for 2 h with the same incubation medium, which now contained 1 mM unlabelled glycerol and the respective thia fatty acids.

Harvesting of cells. The culture dishes were placed on ice after the incubation times indicated. The medium was collected, centrifuged at 2000 rpm for 5 min, and a cell-free aliquot was further treated as explained later. The cells were scraped off the culture dish with a rubber policeman into 2 mL phosphate buffered saline (NaCl/P_i), pH 7.4, containing NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8 mM) and KH_2PO_4 (1.5 mM). The culture dishes were washed once with 2 mL NaCl/P_i. The cell suspensions were centrifuged, and the cells were resuspended in 0.5 or 1.0 mL distilled water and frozen (–20°C). The protein content of each culture dish was determined using bovine serum albumin as standard (18).

Lipid extraction and TLC. The cell suspension was mixed with 20 vol of chloroform/methanol (2:1, vol/vol) (19), 4 vol of NaCl/P_i, pH 2.0, were added and the mixture was allowed to separate into two phases. The organic phase was dried under a stream of nitrogen at 45°C. Medium (1.0 mL) devoid of cellular debris was added to four volumes of chloroform/methanol (2:1, vol/vol) and 2% serum as lipid carrier. The water phase of the medium extract was reextracted once with four volumes of chloroform/methanol (2:1, vol/vol), and the combined organic phases were further treated in the same way as for the cells.

The residual lipid extract was redissolved in 200 μL hexane and separated by TLC using hexane/diethyl ether/acetic acid (80:20:1, by vol) as developing solvent. In other experiments, where it was important to separate diacylglycerol and unesterified cholesterol, hexane/diethyl ether/acetic acid (65:35:1, by vol) followed by hexane as a second developing solvent was used. The TLC foils were cut into scintillation vials and counted in a liquid scintillation spectrometer.

Fatty acid oxidation. Measurement of oxidation of palmitic acid to acid-soluble products (as a determination of the rate of β-oxidation) was performed as described later. Two mL DME medium, containing HEPES (20 mM), [1-^{14}C]palmitic acid (0.2–0.5 μCi/mL, 200 μM), and 200 μM oleic acid or 200 μM tetradecylthioacetic acid was added to cultured hepatocytes. Some cultures also contained 500 μM L-carnitine hydrochloride. The cells were incubated for 1 or 4 h; the medium was collected and centrifuged at 2000 rpm for 5 min. An aliquot (250 μL) was added to 100 μL 6% bovine serum albumin and precipitated with 1.0 mL ice-cold 1 M $HClO_4$. The mixture was centrifuged at 3500 rpm for 10 min, and samples of 500 μL of the supernatants were counted by liquid scintillation.

Statistical analysis. Data in figures are presented as mean ± SD of at least three culture dishes/flasks of hepatocytes, representative of two separate experiments. Statistical analysis was performed on data from three independent experiments

TABLE 1
Effects of 3-Thiadicarboxylic Acid and Tetradecylthioacetic Acid on Incorporation of [^{3}H]Water into Synthesized Lipids by Cultured Rat Hepatocytes[a]

Lipid classes	3-Thiadicarboxylic acid (50 μM)	Tetradecylthioacetic acid (50 μM)
	(% of control)	
Triacylglycerol	76 ± 5[b]	71 ± 7[b]
Diacylglycerol	71 ± 3[b]	44 ± 15[b]
Phospholipids	75 ± 11	83 ± 12
Free fatty acids	83 ± 15	87 ± 8
Cholesterol	60 ± 3[b]	76 ± 11[b]
Cholesteryl ester	52 ± 6[b]	100 ± 6

[a]The cells were incubated for 9 h in the presence of 3 mCi [^{3}H]water/mL, 80 μM bovine serum albumin, and 200 μM oleic acid. Incorporation of radioactivity was determined as described in the Materials and Methods section. Control values from cells incubated with 200 μM oleic acid (cpm/mg cell protein): triacylglycerol, 30700 ± 5200; diacylglycerol, 3350 ± 240; phospholipids, 7300 ± 1550; free fatty acids, 4900 ± 750; cholesterol, 3300 ± 30; and cholesteryl ester, 1110 ± 200. Data represents means ± SD from three independent experiments.

[b]$P < 0.05$ for difference between control cells and cells treated with fatty acid analogues.

when available (Tables 1 and 2). Differences were tested with the Mann-Whitney nonparametric test at 95% confidence interval. The counting efficiency for ^{3}H and ^{14}C was 65 and 92%, respectively, calculated by means of external standards, and quenching was similar in all experiments.

RESULTS

Incorporation of [^{3}H]water into synthesized and secreted lipids. The incorporation of [^{3}H]water into synthesized triacylglycerol, cholesterol, and cholesteryl ester was decreased after nine hours incubation with increasing concentrations of 3-thiadicarboxylic acid (Fig. 1). The strongest inhibitory effect of 3-thiadicarboxylic acid was on cholesterol and cholesteryl ester formation, with 40 and 48% (n = 3, $P < 0.05$) reduction, respectively, at 50 μM of the 3-thia fatty acid. 50 μM tetradecylthioacetic acid reduced [^{3}H]water incorporation into triacylglycerol and cholesterol with 30 and 24% (n = 3, $P < 0.05$), respectively, but no effect was observed on cholesteryl ester formation (Fig. 1, Table 1).

The incorporation of [^{3}H]water into synthesized diacylglycerol also was inhibited by the 3-thia fatty acids. The incorporation was reduced by 55% (n = 3, $P < 0.05$) by 50 μM tetradecylthioacetic acid and by 30% (n = 3, $P < 0.05$) by 50 μM 3-thiadicarboxylic acid compared to the control (Table 1). No inhibition of incorporation of [^{3}H]water into cell-associated monoacylglycerol was found (data not shown).

Increasing concentrations of both fatty acid analogues decreased incorporation of [^{3}H]water into secreted triacylglycerol and cholesteryl ester. 3-Thiadicarboxylic acid also reduced the incorporation into secreted unesterified cholesterol, an effect that was observed only at the highest concentration of tetradecylthioacetic acid examined (Fig. 1).

Incorporation of [^{3}H]glycerol into synthesized and secreted lipids. Tetradecylthioacetic acid (200 μM) and 3-thiadicarboxylic acid reduced the incorporation of tritium-labeled glycerol into cellular diacylglycerol after 1 h of incubation by 35 and 25% (n = 3, $P < 0.05$), respectively (Table 2). The incorporation of [^{3}H]glycerol into synthesized triacylglycerol was only marginally affected in the presence of tetradecylthioacetic acid as compared to control cells (Fig. 2A, Table 2).

In contrast, the incorporation of labeled glycerol into secreted triacylglycerol was significantly reduced by both 3-thia fatty acids (Fig. 2B). Tetradecylthioacetic acid (200 μm) decreased incorporation into secreted triacylglycerol by 50% (n = 3, $P < 0.05$), although 200 μM 3-thiadicarboxylic acid reduced this incorporation only with 37% (n = 3, $P < 0.05$) relative to control cells (Table 2). However, when the cells were labelled with [^{3}H]glycerol for one hour and then chased with unlabelled glycerol for two hours, the 3-thia fatty acids did not reduce the secretion of labelled triacylglycerol compared to control cells (Fig. 2C).

TABLE 2
Effects of 3-Thiadicarboxylic Acid and Tetradecylthioacetic Acid on Incorporation of [^{3}H]Glycerol into Synthesized and Secreted Lipids by Cultured Rat Hepatocytes[a]

Compounds added	Phospholipids synthesized	Diacylglycerol synthesized	Triacylglycerol	
			Synthesized	Secreted
	(% of control)			
3-Thiadicarboxylic acid				
50 μM	94 ± 7	78 ± 12	87 ± 16	76 ± 22
200 μM	93 ± 6	74 ± 8[b]	98 ± 15	63 ± 16[b]
Tetradecylthioacetic acid				
50 μM	103 ± 16	79 ± 22	95 ± 15	75 ± 24
200 μM	97 ± 19	65 ± 19[b]	102 ± 17	48 ± 12[b]

[a]The cells were incubated for 1 h in the presence of 5 μCi [^{3}H]glycerol/.mL, 80 μM bovine serum albumin, and 200 μM oleic acid. Control values from cells incubated with 200 μM oleic acid (cpm/mg cell protein): phospholipids, 34300 ± 5700; diacylglycerol, 3300 ± 280; triacylglycerol, 36200 ± 4900 and 3300 ± 420 for synthesized and secreted, respectively. Data represents means ± SD from three independent experiments.

[b]$P < 0.05$ for difference between control cells and cells treated with fatty acid analogues.

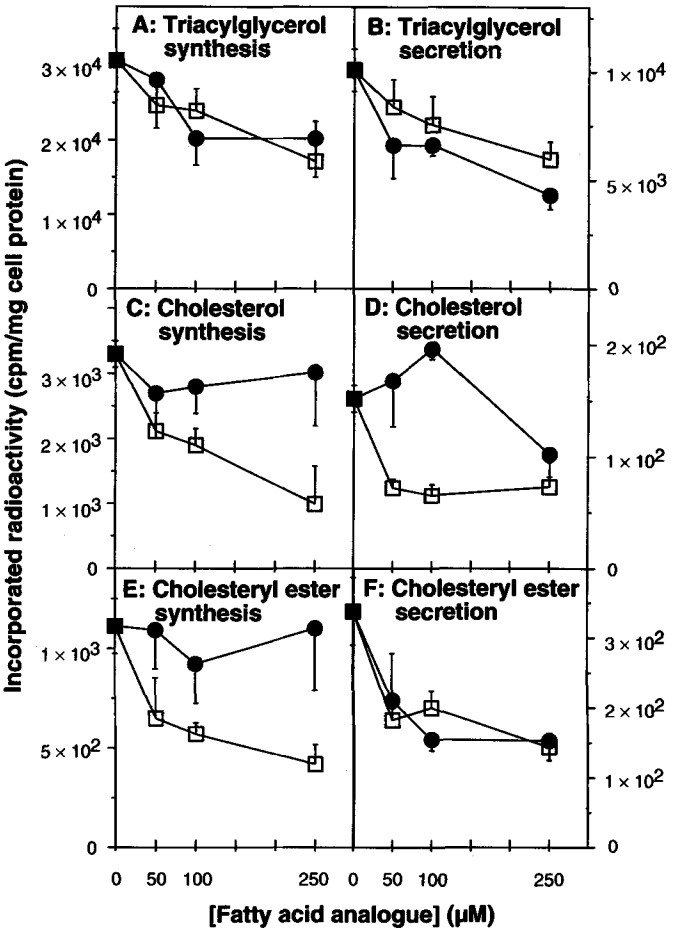


FIG. 1. Dose-dependent effects of 3-thiadicarboxylic acid (□) and tetradecylthioacetic acid (●) on incorporation of [^{3}H]water into synthesized (cell associated + secreted) and secreted lipids by cultured rat hepatocytes. A, Triacylglycerol synthesis; B, triacylglycerol secretion; C, cholesterol synthesis; D, cholesterol secretion; E, cholesteryl ester synthesis; and F, cholesteryl ester secretion. The cells were incubated for nine hours in the presence of 3 mCi [^{3}H]water/mL, 200 μM oleic acid/80 μM bovine serum albumin, and various concentrations of 3-thia fatty acid analogues. Data represent means ± SD for triplicate culture dishes.

Incorporation of labeled water and glycerol into synthesized and secreted lipids in the absence of oleic acid. In the absence of oleic acid, the incorporation of [^{3}H]water into synthesized glycerolipids was only marginally affected (Table 3). The same was observed for synthesized cholesterol and cholesteryl esters (data not shown). Thus, the inhibition of incorporation of [^{3}H]water into synthesized lipids by the sulfur-substituted fatty acids was most pronounced when oleic acid was added to the culture medium (Fig. 1, Table 3). It is also worth noting that, in the absence of oleic acid, the 3-thia fatty acids increased the incorporation of [^{3}H]water into secreted triacylglycerol and phospholipids when compared to no addition (Table 3).

Oxidation of [^{14}C]palmitic acid. The oxidation of [^{14}C]palmitic acid to acid-soluble products was increased nearly fivefold in cultured hepatocytes with increasing concentrations of tetradecylthioacetic acid in the presence of 200 μM oleic acid (Fig. 3). Tetradecylthioacetic acid increased the oxidation of [^{14}C]palmitic acid significantly more than oleic acid when these fatty acids were compared at equal concentrations (Fig. 4). 3-Thiadicarboxylic acid stimulated the fatty acid oxidation only slightly (Fig. 3). The rate of fatty acid oxidation was influenced by the addition of L-carnitine to the hepatocytes, but the relative stimulation by tetradecylthioacetic acid was similar irrespective of the addition of L-carnitine. None of the 3-thia fatty acids increased the peroxisomal fatty acyl–CoA oxidase activity when hepatocytes were incubated for up to 12 h (data not shown).

DISCUSSION

The hypolipidemic effects of non-β-oxidizable sulfur-substituted fatty acid analogues in rats *in vivo* are well documented (1–4). In this study we have demonstrated that these analogues also promoted lower synthesis and secretion of lipids in cultured rat hepatocytes (Fig. 1, Table 1). The two analogues had similar effects on the metabolism of triacylglycerol and phospholipids, although the metabolism of cholesterol was affected differently.

Using different radiolabeled precursors, the two 3-thia fatty acids showed variable effects on the synthesis of triacylglycerol and phospholipids (Tables 1 and 2). In hepatocytes labeled with [^{3}H]water, the synthesis and secretion of triacylglycerol were reduced to a similar extent (Fig. 1), and in experiments with [^{3}H]glycerol only secretion of triacylglycerol was significantly reduced (Fig. 2). It is possible that the different time of incubation, nine hours in experiments with [^{3}H]water and one hour in experiments with [^{3}H]glycerol, could be responsible for this discrepancy. In experiments where hepatocytes have been incubated with radiolabeled fatty acids for up to four hours, reduced secretion of triacylglycerol has been found without any significant effect on its synthesis (6,11).

It is noteworthy that the incorporation of labeled precursors into synthesized diacylglycerol was reduced to a greater extent than the incorporation into triacylglycerol and phospholipids, even in the short-term experiment with [^{3}H]glycerol (Tables 1 and 2). As the the sulfur-substituted fatty acids had only small effects on incorporation of [^{3}H]water into cellular monoacylglycerols (data not shown), these data suggest that 3-thia fatty acids decrease triacylglycerol synthesis by affecting some steps just prior to the formation of diacylglycerol. Reduced activity of phosphatidate phosphohydrolase has been shown to correlate with diminished triacylglycerol synthesis in some studies (20–22), and reduced triacylglycerol levels and phosphatidate phosphohydrolase activity previously have been found in livers from rats fed 3-thia fatty acids (3).

The effects of the fatty acid analogues have been studied in the presence of 200 μM oleic acid in the medium. It is well established that addition of up to 1 mM oleic acid to cultured hepatocytes stimulates synthesis and secretion of triacylglycerol, but the secretion of total apoproteinB is not stimulated

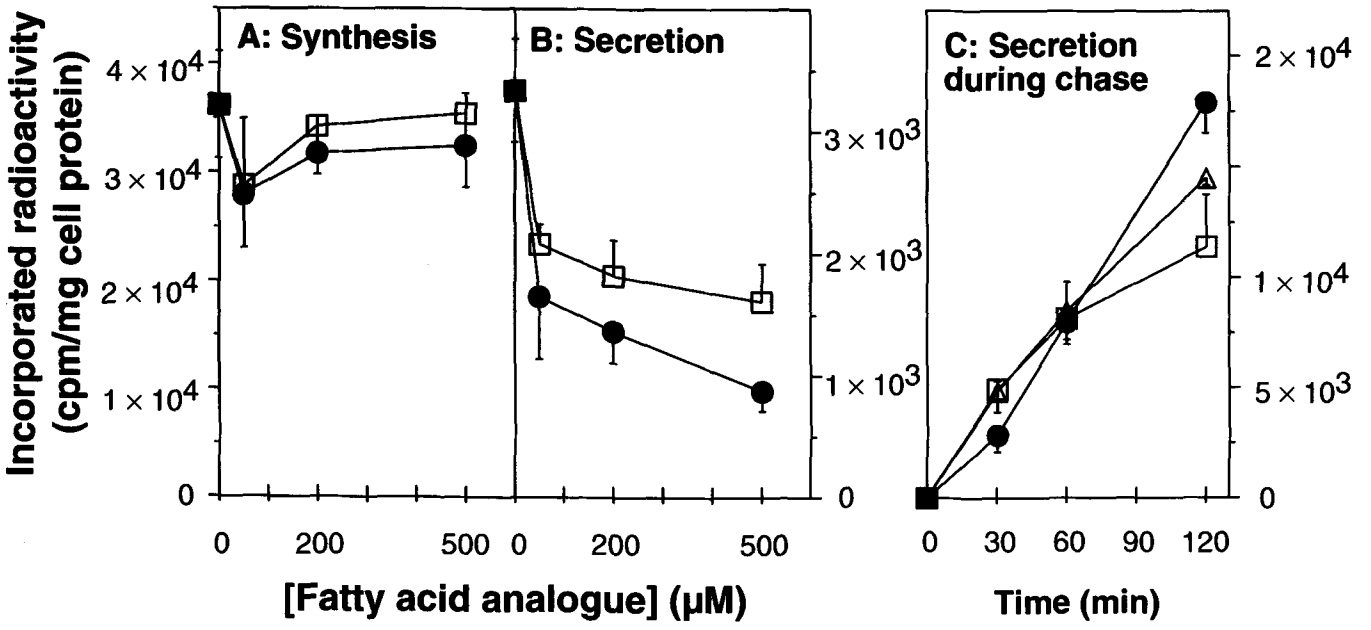


FIG. 2. Dose-dependent effects of 3-thiadicarboxylic acid (□) and tetradecylthioacetic acid (●) on incorporation of [^{3}H]glycerol into synthesized and secreted triacylglycerol by cultured rat hepatocytes. A, Triacylglycerol synthesis; B, triacylglycerol secretion; C, triacylglycerol secretion when hepatocytes were chased with 1 mM unlabelled glycerol. (△) Control cells. The cells were incubated for one hour in the presence of of 5 μCi [^{3}H]glycerol/mL (25 μM), 200 μM oleic acid/80 μM bovine serum albumin, and various concentrations of 3-thia fatty acid analogues. In C, 200 μM of 3-thia fatty acids were added. Data represent means ± SD for triplicate culture dishes.

TABLE 3
Incorporation of [^{3}H]Water into Synthesized and Secreted Glycerolipids with Different Combinations of Fatty Acids Added to Cultured Rat Hepatocytes[a]

Compounds added	Phospholipids		Triacylglycerol		Diacylglycerol
	Synthesized	Secreted	Synthesized	Secreted	Synthesized
			(% of control)		
Oleic acid	82 ± 19	164 ± 69	121 ± 16	185 ± 17	119 ± 7
3-Thiadicarboxylic acid	86 ± 18	425 ± 123	108 ±14	193 ± 35	95 ± 18
3-Thiadicarboxylic acid + oleic acid	43 ± 16	167 ± 57	67 ± 21	109 ± 14	52 ± 25
Tetradecylthioacetic acid	75 ± 8	457 ± 64	112 ± 15	155 ± 29	87 ± 10
Tetradecylthioacetic acid + oleic acid	53 ± 10	141 ± 75	80 ± 20	79 ± 11	46 ± 12

[a]The cells were incubated for 9 h in the presence of 3 mCI [^{3}H]water/mL and 80 μM bovine serum albumin. The various fatty acids were present in the following concentrations: oleic acid, 200 μM; 3-thiadicarboxylic acid, 250 μM; and tetradecylthioacetic acid, 250 μM. Incorporation of radioactivity was determined as described in the Materials and Methods section. Control values from cells incubated with 80 μM bovine serum albumin (cpm/mg cell protein): phospholipids, 8900 ± 1600, and 84 ± 35 for synthesized and secreted, respectively; triacylglycerol, 25300 ± 4500 and 5450 ± 1050 for synthesized and secreted, respectively; diacylglycerol, 2810 ± 640. Data represents means ± SD for triplicate culture flasks.

(23–27). The data in Table 3 suggest that the effects of 3-thia fatty acids on the lipid metabolism in hepatocytes are dependent on the presence of oleic acid in the medium. When hepatocytes were cultured in the absence of oleic acid, the fatty acid analogues rather stimulated the incorporation of [^{3}H]water into synthesized and secreted triacylglycerol (Table 3), mimicking the effect of oleic acid. It is possible that 3-thia fatty acids may have dual effects dependent on the actual fluxes of lipid metabolites. Without oleic acid in the medium, the flux of fatty acids into glycerolipids may be low. In this situation the fatty acid analogues act in the same way as ordinary fatty acids, increasing triacylglycerol synthesis and secretion. When the flux of fatty acids is increased due to the addition of oleic acid, the analogues may act by regulating rate-limiting enzymes. We have observed that the activity of lipogenic enzymes is decreased in the liver of rats treated with 3-thia fatty acids, and that this decrease is more pronounced when the rate of lipogenesis is high (4,28).

The effects of 3-thiadicarboxylic acid and tetradecyl-

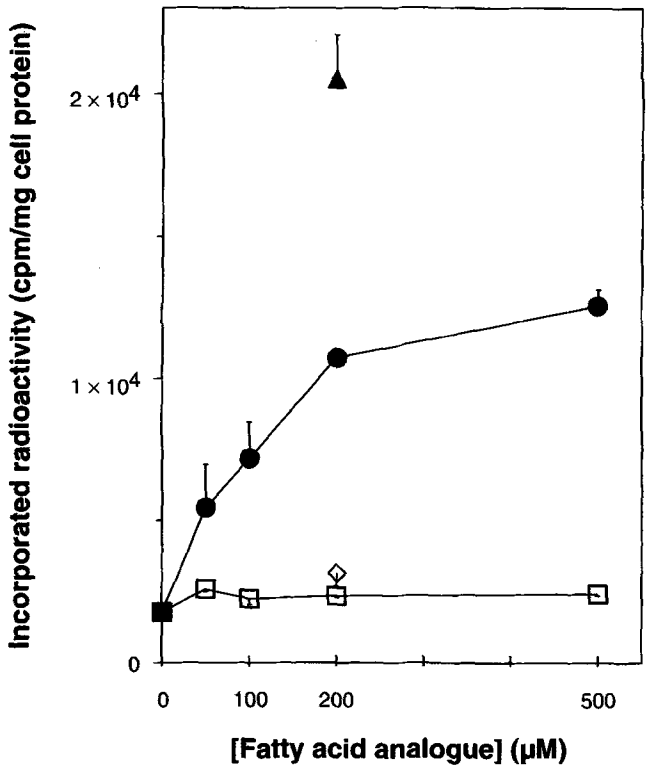


FIG. 3. Effects of fatty acid analogues on the oxidation of [^{14}C]palmitic acid to acid-soluble products by cultured rat hepatocytes. The cells were incubated for one hour with 0.2 μCi [^{14}C]palmitic acid, 200 μM, and 200 μM oleic acid in addition to tetradecylthioacetic acid (●); tetradecylthioacetic acid and 500 μM L-carnitine (▲); 3-thiadicarboxylic acid (□); 3-thiadicarboxylic acid and 500 μM L-carnitine (◇). Data represent means ± SD for triplicate culture dishes.

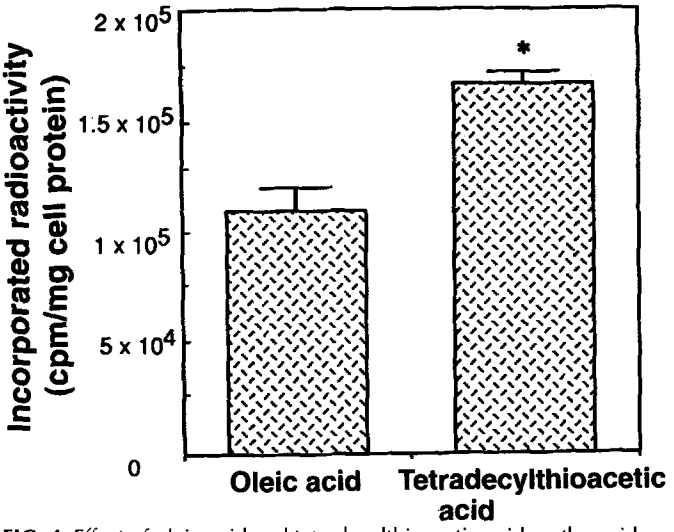


FIG. 4. Effect of oleic acid and tetradecylthioacetic acid on the oxidation of [^{14}C]palmitic acid to acid-soluble products by rat hepatocytes. The cells were incubated for four hours with 0.5 μCi [^{14}C]palmitic acid (200 μM) and L-carnitine (500 μM) in addition to either oleic acid (200 μM) or tetradecylthioacetic acid (200 μM). Data represent means ± SD of three independent experiments.*$P < 0.05$ for difference between cells incubated with oleic acid and tetradecylthioacetic acid.

thioacetic acid on cultured hepatocytes were similar in many aspects. However, some noteworthy exceptions with regard to β-oxidation of fatty acids and cholesterol metabolism merit further considerations.

Adding tetradecylthioacetic acid to cultured hepatocytes increased the oxidation of [^{14}C]palmitic acid compared to the addition of oleic acid (Fig. 4). This difference may be partly due to the non-β-oxidizability of tetradecylthioacetic acid (8–10), allowing it to compete with the available free fatty acids for esterification reactions (10). Compared to the addition of [^{14}C]palmitic acid and oleic acid, which both may be β-oxidized, relatively more [^{14}C]palmitic acid will be β-oxidized when tetradecylthioacetic acid and [^{14}C]palmitic acid are added together. Due to competition between tetradecylthioacetic acid and ordinary fatty acids, it is conceivable that when hepatocytes are cultured in the presence of this 3-thia fatty acid, ordinary fatty acids will be diverted into β-oxidation instead of esterification, especially into phospholipids (10). It also has been found that tetradecylthioacetic acid feeding rapidly decreases the level of malonyl–CoA in rat liver (29), which may further contribute to increased β-oxidation through the well-known regulation of carnitine palmitoyltransferase by malonyl–CoA (30,31). Previously, we have found an increased capacity for mitochondrial β-oxidation in liver of rats fed tetradecylthioacetic acid (2,3,5). The availability of ordinary fatty acids may therefore limit the synthesis of triacylglycerol in the hepatocytes.

The reduced output of triacylglycerol by tetradecylthioacetic acid may be related to the stimulation of fatty acid oxidation. It has been shown previously that inhibitors of fatty acid synthesis increased fatty acid oxidation and ketogenesis, accompanied by a decreased secretion of triacylglycerol (32,33). Recently, it has been found that the secretion of triacylglycerol from hepatocytes is less affected by tetradecylthioacetic acid when fatty acid oxidation is inhibited (6). 3-Thiadicarboxylic acid, however, did not significantly increase the oxidation of [^{14}C]palmitic acid when incubated with cultured hepatocytes (Fig. 4), in contrast to what has been observed in rat liver (4). Nevertheless, the secretion of triacylglycerol was reduced (Fig. 1), even after one hour of incubation (Fig. 2). This points to some possible differences in their mechanism of action.

The rate of secretion of triacylglycerol also was generally reduced to a greater extent with tetradecylthioacetic acid than with 3-thiadicarboxylic acid. This is seen in hepatocytes labelled with [^{3}H]water, with (Fig.1) and without oleic acid in the medium (Table 3), and also was observed with [^{3}H]glycerol (Fig. 2B) as radioactive precursor. When pulse-chase experiments were performed, none of the 3-thia fatty acids reduced the secretion of triacylglycerol (Fig. 2C), indicating that the fatty acid analogues do not affect the secretion process *per se*, but rather some step(s) prior to the complete assembly of lipoprotein particles.

Cholesterol synthesis and esterification were clearly inhibited by 3-thiadicarboxylic acid, whereas tetradecylthioacetic acid marginally affected the incorporation of [^{3}H]water into

cholesterol and cholesteryl ester (Fig. 1, Table 1). In rat liver *in vivo*, it has been observed that both fatty acid analogues inhibited the activity of the rate-limiting enzyme in the biosynthesis cholesterol, HMG–CoA reductase, although not to the same extent (4,11). It is thus likely that the inhibition of cholesterogenesis by 3-thiadicarboxylic acid is primarily due to reduced HMG–CoA reductase activity. This inhibition also may account for the cholesterol-lowering effect of the fatty acid analogue, as the incorporation of [^{3}H]water into secreted cholesterol and cholesteryl ester was reduced to the same extent as the synthesis (Fig. 1). The reason why tetradecylthioacetic acid inhibited HMG–CoA reductase *in vivo* (11), while no inhibition of cholesterogenesis was seen *in vitro*, is not obvious. In contrast to 3-thiadicarboxylic acid, this analogue decreased the secretion of cholesteryl ester without a concomitant reduction in its synthesis (Fig. 1).

The intracellular concentrations of 3-thia fatty acids in rat liver *in vivo* and in hepatocytes cultured *in vitro* are not known, but different effects of these fatty acid analogues *in vitro* and *in vivo* might possibly be related to differences in intracellular concentrations in these two situations. Apart from these considerations, it is noteworthy that even if the biological effects of the 3-thia fatty acids are very similar *in vivo*, the results obtained with regard to β-oxidation, triacylglycerol, and cholesterol metabolism when these fatty acids are added to hepatocytes *in vitro* seem to indicate that 3-thiadicarboxylic acid and tetradecylthioacetic acid may have different mechanisms of action.

ACKNOWLEDGMENTS

The authors are grateful to Randi Sandvik, Svein Krüger and Anne R. Alvestad for excellent assistance. The work was supported by grants from The Norwegian Council on Cardiovascular Disease (NCCD), Pronova AS, Nordisk Insulinfond, Fondet for Hjertforskning, University of Bergen, The Norwegian Research Council for Science and the Humanities, Freia Chokoladefabrik's Medicinske Forskningsfond, and Anders Jahres Medisinske Fond. J. Skorve is research fellow of NCCD.

REFERENCES

1. Berge, R.K., Aarsland, A., Kryvi, H., Bremer, J. and Aarsaether, N. (1989) Alkylthio Acetic Acids (3-thia fatty acids)—A New Group of Non-β-Oxidizable Peroxisome-Inducing Fatty Acid Analogues—II. Dose-Response Studies on Hepatic Peroxisomal- and Mitochondrial Changes and Long-Chain Fatty Acid Metabolizing Enzymes in Rats, *Biochem. Pharmacol. 38*, 3969–3979.
2. Aarsland, A., Aarsaether, N., Bremer, J., and Berge, R.K. (1989) Alkylthioacetic Acids (3-thia fatty acids) as Non-β-Oxidizable Fatty Acid Analogues: A New Group of Hypolipidemic Drugs. III. Dissociation of Cholesterol- and Triglyceride-Lowering Effects and the Induction of Peroxisomal β-Oxidation, *J. lipid Res. 30*, 1711–1718.
3. Skorve, J., Asiedu, D., Rustan, A.C., Drevon, C.A., Al-Shurbaji, A., and Berge, R.K. (1990) Regulation of Fatty Acid Oxidation and Triglycride and Phospholipid Metabolism by Hypolipidemic Sulfur-Substituted Fatty Acid Analogues, *J. Lipid Res. 31*, 1627–1635.
4. Skorve, J., Al-Shurbaji, A., Asiedu, D., Björkhem, I., Berglund, L., and Berge, R.K. (1993) On the Mechanism of the Hypolipidemic Effect of Sulfur-Substituted Hexa-Decanedioic Acid (3-thiadicarboxylic acid) in Normolipidemic Rats, *J. Lipid Res. 34*, 1177–1185.
5. Asiedu, D.K., Skorve, J., Willumsen, N., Demoz, A., and Berge, R.K. (1993) Early Effects on Mitochondrial and Peroxisomal β-Oxidation by the Hypolipidemic 3-Thia Fatty Acids in Rat Livers, *Biochim. Biophys. Acta. 1166*, 73–76.
6. Skrede, S., Bremer, J., Berge, R.K., and Rustan, A.C. (1994) Stimulation of Fatty Acid Oxidation by a 3-Thia Fatty Acid Reduces Triacylglycerol Secretion in Rat Hepatocytes, *J. Lipid Res. 35*, 1395–1404.
7. Aarsland, A., and Berge, R.K. (1991) Peroxisome Proliferating Sulphur- and Oxy-Substituted Fatty Acid Analogues Are Activated to Acyl Coenzyme A Thioesters, *Biochem. Pharmacol. 41*, 53–61.
8. Asiedu, D.K., Demoz, A., Skorve, J., Grav, H.J. and Berge, R.K. (1995) Acute Modulation of Rat Hepatic Lipid Metabolism by Sulphur-Substituted Fatty Acid Analogues, *Biochem. Pharmacol. 49*, 1013–1022.
9. Skrede, S., Narce, M., Bergseth, S., and Bremer, J. (1989) The Effects of Alkyl Thioacetic Acids (3-thia fatty acids) on Fatty Acid Metabolism in Isolated Hepatocytes, *Biochim. Biophys. Acta. 1005*, 296–302.
10. Hvattum, E., Grav, H., and Bremer, J. (1993) Hormonal and Substrate Regulation of 3-Thia Fatty Acid Metabolism in Morris 7800 C1 Hepatoma Cells, *Biochem. J. 294*, 917–921.
11. Asiedu, D., Al-Shurbaji, A., Rustan, A.C., Björkhem, I., Berglund, L., and Berge, R.K. (1995) Hepatic Fatty Acid Metabolism as a Determinant of Plasma and Liver Triacylglycerol Levels. Studies on Tetradecylthioacetic Acid and Tetradecylthiopropionic Acids, *Eur. J. Biochem. 227*, 715–722.
12. Nossen, J.Ø., Rustan, A.C., Gloppestad, S.H., Målbakken, S., and Drevon, C.A. (1986) Eicosapentaenoic Acid Inhibits Synthesis and Secretion of Triacylglycerols by Cultured Rat Hepatocytes, *Biochim. Biophys. Acta 879*, 56–65.
13. Rustan, A.C., Nossen, J.Ø., Christiansen, E.N., and Drevon, C.A. (1988) Eicosapentaenoic Acid Reduces Hepatic Synthesis and Secretion of Triacylglycerol by Decreasing the Activity of Acyl-Coenzyme A: 1,2-Diacylglycerol Acyltransferase, *J. Lipid Res. 29*, 1417–1426.
14. Rustan, A.C., Nossen, J.Ø., Osmundsen, H., and Drevon, C.A. (1988) Eicosapentaenoic Acid Inhibits Cholesterol Esterification in Cultured Parenchymal Cells and Isolated Microsomes from Rat Liver, *J. Biol. Chem. 263*, 8126–8132.
15. Aarsland, A., Berge, R.K., Bremer, J., and Aarsæther, N. (1990) The Hypolipidemic Peroxisome-Proliferating Drug, *bis*(carboxymethylthio)-1.10 Decane, a Dicarboxylic Metabolite of Tiadenol, Is Activated to an Acylcoenzyme A Thioester, *Biochim. Biophys. Acta 1033*, 176–183.
16. Spydevold, Ø., and Bremer, J. (1989) Induction of Peroxisomal β-Oxidation in 7800 Morris Hepatome Cells in Steady State by Fatty Acids and Fatty Acid Analogues, *Biochim. Biophys. Acta. 1003*, 72–79.
17. Seglen, P.O. (1976) Preparation of Isolated Rat Liver Cells, in *Methods in Cell Biology* (Prescott, D.M., ed.) Vol. 13, Academic Press, New York, pp. 39–83.
18. Bradford, M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*, 248–254.
19. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem.* 226, 497–509.
20. Marsh, J.B., Topping, D.L., and Nestel, P.J. (1987) Comparative effects of Dietary Fish Oil and Carbohydrate on Plasma

Lipids and Hepatic Activities of Phosphatidate Phosphohydrolase, Diacylglycerol Acyltransferase and Neutral Lipase Activities in the Rat, *Biochim. Biophys. Acta 922*, 239–243.
21. Halminski, M.A., Marsh, J.B., and Harrison, E.H. (1991) Differential Effects of Fish Oil, Safflower Oil and Palm Oil on Fatty Acid Oxidation and Glycerolipid Synthesis in Rat Liver, *J. Nutr. 121*, 1554–1561.
22. Surette, M.E., Whelan, J., Broughton, K.S., and Kinsella, J.E. (1992) Evidence for the Mechanisms of the Hypotriglyceridemic Effect of n-3 Polyunsaturated Fatty Acids, *Biochim. Biophys. Acta 1126*, 199–205.
23. Davis, R.A., and Boogaerts, J.R. (1982) Intrahepatic Assembly of Very Low Density Lipoproteins. Effect of Fatty Acids on Triacylglycerol and Apolipoprotein Synthesis, *J. Biol. Chem. 257*, 10908–10913.
24. Gibbons, G.F. (1990) Assembly and Secretion of Hepatic Very Low Density Lipoprotein, *Biochem. J. 268*, 1–13.
25. Duerden, J.M., Marsh, B., Burnham, F.J., and Gibbons, G.F. (1990) Regulation of Hepatic Synthesis and Secretion of Cholesterol and Glycerolipids in Animals Maintained in Different Nutritional States, *Biochem. J. 271*, 761–766.
26. Gibbons, G.F., and Burnham, F.J. (1991) Effect of Nutritional State on the Utilization of Fatty Acids for Hepatic Triacylglycerol Synthesis and Secretion as Very Low Density Lipoprotein, *Biochem. J. 275*, 87–92.
27. Dixon, J.L., and Ginsberg, H.N. (1993) Regulation of Hepatic Secretion of Apo-Lipoprotein B-Containing Lipoproteins: Information Obtained from Cultured Liver Cells, *J. Lipid Res. 34*, 167–179.
28. Skorve, J., and Berge, R.K. (1993) The Hypocholesterolemic Effect of Sulfur-Substituted Fatty Acid Analogues in Rats Fed a High Carbohydrate Diet, *Biochim. Biophys. Acta 1167*, 175–181.
29. Skrede, S., and Bremer, J. (1993) Acylcarnitine Formation and Fatty Acid Oxidation in Hepatocytes from Rats Treated with Tetradecylthioacetic Acid (a 3-thia fatty acid), *Biochim. Biophys. Acta. 1167*, 189–196.
30. McGarry, J.D., Mannaerts, G.P. and Foster, D.W. (1977) A Possible Role for Malonyl-CoA in the Regulation of Hepatic Fatty Acid Oxidation and Ketogenesis, *J. Clin. Invest. 60*, 265–270.
31. McGarry, J.D., and Foster, D.W. (1980) Regulation of Hepatic Fatty Acid Oxidation and Ketone Body Production, *Annu. Rev. Biochem. 49*, 395–420.
32. Fukuda, N., and Ontko, J.A. (1984) Interactions Between Fatty Acid Synthesis, Oxidation, and Esterification in the Production of Triglyceride-Rich Lipoproteins by the Liver, *J. Lipid Res. 25*, 831–842.
33. Yamamoto, M., Fukuda, N., Triscari, J., Sullivan, A.C., and Ontko, J.A. (1985) Decreased Hepatic Production of Very Low Density Lipoproteins Following Activation of Fatty Acid Oxidation by Ro 22-0654, *J. Lipid Res. 26*, 1196–1204.

[Received September 30, 1994, and in final revised form June 19, 1995; Revision accepted August 2, 1995]

Enzymatic Hydrolysis of Long-Chain *N*-Heterocyclic Fatty Esters

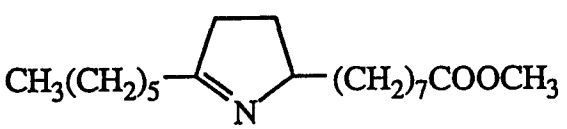
Marcel S.F. Lie Ken Jie* and M.S.K. Syed-Rahmatullah

Department of Chemistry, The University of Hong Kong, Hong Kong

ABSTRACT: Incubation of a 1-pyrroline ester [viz. methyl 8-(5-hexyl-1-pyrroline-2-yl)octanoate, **1**] with bakers' yeast *(Saccharomyces cerevisiae)* gave the corresponding free fatty acid (**1a**, 52%). The C=N bond of the 1-pyrroline was not reduced by the yeast. Complete hydrolysis of compound **1** was successful using lipase of *Candida cylindracea* (CCL) or Lipolase *(Rhizomucor miehei)* under stirred or ultrasound condition. Fatty esters containing a pyrrolidine [viz. methyl 8-(*cis/trans*-5-hexyl-pyrrolidine-2-)octanoate, **2**] or *N*-methyl pyrrolidine [viz. methyl 8-(*cis*-5-hexyl-*N*-methyl-pyrrolidine-2-)octanoate, **3**] system in the alkyl chain were not hydrolyzed by either CCL or Lipolase, unless conducted in an ultrasonic bath. The hydrolytic activities of the enzymes appeared to be strongly affected by the stereochemistry of the *N*-heterocyclic ring system. Chemical hydrolysis of compounds **1–3** gave the corresponding fatty acid *N*-HCl salts.

Lipids 30, 995–999 (1995).

2,5-Dialkyl and 2,5-dialkenyl substituted 1-pyrrolines and pyrrolidines have been found to occur as venomous constituents of the fire ants *Solenopsis* and *Monomorium* species (1–3). These *N*-heterocyclic compounds possess antibacterial, antifungal, phytotoxic, insecticidal, haemolytic, and necrotoxic properties (4–8). However, long-chain fatty acids containing *N*-heterocyclic rings have not been found in nature. We have reported the synthesis of a novel 1-pyrroline fatty acid ester isomer, viz. methyl 8-(5-hexyl-1-pyrroline-2-yl)octanoate (**1**) from methyl *iso*-ricinoleate (9). This *N*-heterocyclic fatty ester and its derivatives are currently being investigated for their antifungal and antimicrobial properties for which the free acids are also required (Scheme 1).



(1)

SCHEME 1

*To whom correspondence should be addressed at the Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong.

Abbreviations: CCL, *Candida cylindracea*; HR–MS, high-resolution mass spectra; NMR, nuclear magnetic resonance spectroscopy.

Fatty esters containing the 1-pyrroline nucleus are readily transformed to the saturated *cis*- and *trans*-pyrrolidine (**2**) isomers by reduction of the C=N bond with sodium borohydride (9). *N*-Methylation and subsequent sodium borohydride reduction of compound **1** yields a mixture of *cis*- and *trans*-*N*-methyl pyrrolidine esters from which the *cis*-isomer (**3**) is separated by silica chromatography (10).

Bakers' yeast *(Saccharomyces cerevisiae)* is widely used for reducing carbonyl functions where the oxidoreductase of the yeast is activated. This yeast is also used for the hydrolysis of esters due to the presence of ester hydrolase (11). In most enzymatic reactions, mild and neutral pH media are employed. We have chosen bakers' yeast to investigate the hydrolyzing activity and the effect of the oxidoreductase toward the C=N bond of the 1-pyrroline ring system.

The lipases from *Candida cylindracea* (CCL) yeast and *Rhizomucor miehei* fungus (Lipolase™) show optimum hydrolytic activities when experiments are conducted at pH 7.0 in potassium phosphate buffer (12–14). Lipolase is a thermostable lipase and tolerates temperatures up to 60°C, while the optimum temperature in the case of CCL is 37–40°C.

In this paper we compare the results of the hydrolysis of *N*-heterocyclic fatty esters using bakers' yeast and various lipases in neutral media with those obtained by KOH hydrolysis followed by acidification with HCl. The physical properties of the free fatty acids are also compared to those of the corresponding *N*-HCl-salts obtained by chemical hydrolysis.

MATERIALS AND METHODS

The *N*-heterocyclic fatty esters, viz. methyl 8-(5-hexyl-1-pyrroline-2-yl)octanoate (**1**), methyl 8-(*cis/trans*-5-hexyl-pyrrolidine-2-)octanoate (**2**), and methyl 8-(*cis*-5-hexyl-*N*-methyl-pyrrolidine-2-)octanoate (**3**) were synthesized from methyl *iso*-ricinoleate according to procedures described elsewhere (9,10). Methyl 8-(5-hexyl-1*H*-pyrrole-2-)octanoate (**4**) was obtained by cyclodehydration reaction of methyl 9,12-dioxostearate as described elsewhere (15). Lipolase powder (one of the two forms of lipases from *R. miehei*, activity, 100 KLU/g) was a gift from Novo Nordisk A.S. (Hong Kong). One kilo lipase unit (KLU) corresponds to 1 μmol butyric acid formed from tributyrin per min under the conditions specified by the manufacturer. Lipase from CCL (also known

Copyright © 1995 by AOCS Press

as *C. rugosa*) (Type VII, activity 905 U/mg) and bakers' yeast (*S. cerevisiae*,YSC type II) were purchased from Sigma Chemical Co. (St. Louis, MO). One unit of lipase will hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in 1 h at pH 7.2 at 37°C using olive oil as the substrate. Thin-layer and column chromatographic, infrared, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopic analyses were performed as described elsewhere (9). Gas–liquid chromatography was conducted on a Hewlett-Packard (Hewlett-Packard, Palto Alto, CA) model 5890 gas chromatograph fitted with a fused silica capillary column (30 m × 0.5 mm, Supelcowax™ 10 stationary phase, 1.0 μm film thickness; Supelco, Bellefonte, PA) using nitrogen (2 mL/min) as the carrier gas with temperature programmed from 160–240°C at a rate of 5°C/min. The injector and detector (flame-ionization) temperatures were maintained at 230 and 280°C, respectively. A Branson model 3200 (47 kHz) ultrasonic cleaning water bath (Branson Ultrasonic, Danbury, CT) was used for reactions under ultrasound conditions. High-resolution mass spectra (HR–MS) were recorded on a Finnigan Mat 95 mass spectrometer (Finnigan Mat Corp., San Jose, CA).

Enzymatic hydrolysis of the N*-heterocyclic fatty esters: (a) Hydrolysis of methyl 8-(5-hexyl-1-pyrroline-2-yl)-octanoate* (**1**) *using bakers' yeast* (S. cerevisiae). To a fermenting mixture of bakers' yeast (10 g), D-glucose (7.6 g), and water (170 mL) in a 250-mL round-bottomed flask at 30°C, 1-pyrroline fatty ester (**1**, 1.0 g, 3.24 mmol) was added and the mixture was stirred for 3 d. The mixture was extracted with diethyl ether (2 × 100 mL). The ethereal extract was washed with water (2 × 50 mL) and dried (Na_2SO_4). Evaporation of solvent under reduced pressure gave a crude product (0.9 g). Silica gel column chromatographic separation using a mixture of light petroleum (b.p. 40–60°C) and diethyl ether (1:1, vol/vol) gave the unreacted ester (0.4 g) followed by elution with dichloromethane to yield 1-pyrroline fatty acid (**1a**) (0.5 g, 52%). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.20–1.45 (*m*, 14H, CH_2), 1.45–1.80 (*m*, 6H, CH_2), 2.00–2.15 (*m*, 2H, ring-CH_2, 10-*H*), 2.27 (*t*, 2H, 2-*H*), 2.41 (*t*, 2H, CH_2, 13-*H*), 2.51 (*m*, 2H, ring-CH_2, 11-*H*), 4.03 (*m*, 1H, C*H*, 9-*H*) and 10.59 (*s*, 1H, COO*H*); ^{13}C NMR ($CDCl_3$, ppm): 14.04 (C-18), 22.51 (C-17), 25.32 (C-3), 26.24 (C-7), 26.45 (C-14), 27.92 (C-10), 29.06, 29.23, 29.29, 29.49, 31.49, 31.60 (C-16), 33.07 (C-2), 35.11 (C-13), 35.88 (C-11), 36.75 (C-8), 70.96 (C-9), 177.57 (C-12, *C*=N), and 180.39 (C-1, *C*OOH).

(b) Hydrolysis of methyl 8-(5-hexyl-pyrroline-2-yl)octanoate (**1**) *using lipase of CCL.* A mixture of 1-pyrroline ester (**1**, 0.2 g, 0.64 mmol), CCL lipase (40 mg), and potassium phosphate buffer (pH 7.0, 2 mL) was stirred at 40°C for 16 h. Diethyl ether (2 mL) was added. The mixture was centrifuged and the ethereal phase was separated. The aqueous phase was reextracted with diethyl ether (2 × 2 mL). The combined ethereal extract was washed with water and evaporated under a stream of nitrogen. An azeotropic mixture containing benzene/methanol/chloroform (1:1:1, by vol) (1 mL) was added, and the mixture was further evaporated under a stream of nitrogen to give 1-pyrroline fatty acid (**1a**) (0.19 g, 100%).

(c) Hydrolysis of methyl 8-(5-hexyl-pyrroline-2-yl)octanoate (**1**) *using Lipolase under silent or ultrasound condition.* A mixture of 1-pyrroline ester (**1**, 0.2 g, 0.64 mmol), Lipolase (40 mg), and potassium phosphate buffer (pH 7.0, 2 mL) was stirred at 60°C for 36 h. Isolation of the product yielded the corresponding free fatty acid **1a** (0.19 g, 100%). When the same reaction mixture was incubated in a Bransonic ultrasound cleaning bath for 3 h at a bath temperature of 57–60°C, isolation of the reaction product furnished the corresponding free fatty acid (100% yield).

*(d) Hydrolysis of methyl 8-(*cis/trans*-5-hexyl-pyrrolidine-2-)octanoate* (**2**) *using lipase of CCL or Lipolase under silent or ultrasound condition.* A mixture of compound **2** (0.2 g, 0.64 mmol), CCL lipase (100 mg), and potassium phosphate buffer (pH 7.0, 2 mL) was stirred at 40°C for 7 d. Extraction of the reaction mixture with diethyl ether (3 × 2 mL) gave unreacted starting ester **2** quantitatively. The same reaction was carried out using Lipolase (40 mg) as the biocatalyst at 55°C for 24 h. Isolation of the mixture gave unreacted ester **2** quantitatively.

A mixture of compound **2** (0.2 g, 0.64 mmol), Lipolase (50 mg), and potassium phosphate buffer (pH 7.0, 2 mL) in a screw-capped test tube was incubated in a Branson ultrasonic cleaning bath at 57–65°C for 48 h. The mixture was extracted with diethyl ether. Silica gel column chromatographic separation of the isolated product using a mixture of light petroleum and diethyl ether (1:1, vol/vol) (30 mL) followed by a mixture of dichloromethane and methanol (1:1, vol/vol) (50 mL) gave unhydrolyzed ester (80 mg) and 8-(*cis/trans*-5-hexyl-pyrrolidine-2-)octanoic acid (**2a**) (97 mg, 51%); ^{1}H NMR ($CDCl_3$, δ): 0.89 (*t*, 3H, CH_3), 1.20–1.60 (*m*, 22H, CH_2), 1.80 (*m*, 2H, ring CH_2 *trans*-isomer), 1.92 (*m*, 2H, ring CH_2 *cis*-isomer), 2.30 (*t*, 2H, 2-*H*), 2.90 (*s*, 1H, N-*H*), 2.90 (*m*, 2H, ring C-*H cis*-isomer), 3.12 (*m*, 2H, ring C-*H trans*-isomer), 3.66 (*s*, 3H, $COOCH_3$), and 10.2 (*s*, 1H, COO*H*).

*(e) Hydrolysis of methyl 8-(*cis*-5-hexyl-*N*-methyl-pyrrolidine-2-)octanoate* (**3**) *using Lipolase under stirred or ultrasound condition.* A mixture of compound **3**, Lipolase (40 mg), and phosphate buffer (pH 7.0, 2 mL) was stirred at 60°C for 24 h. Isolation of the reaction mixture gave unreacted starting ester **3** quantitatively.

A mixture of compound **3** (0.1 g, 0.31 mmol), Lipolase (20 mg), and potassium phosphate buffer (pH 7.0, 2 mL) in a screw-capped test tube was sonicated in a Branson ultrasonic cleaning bath for 24 h at 57–60°C. The mixture was extracted with diethyl ether. Silica gel column chromatographic separation of the isolated product using a mixture of light petroleum and diethyl ether (1:1, vol/vol) (30 mL) followed by a mixture of dichloromethane and methanol (1:1, vol/vol) (50 mL) gave unhydrolyzed ester (20 mg) and 8-(*cis*-5-hexyl-*N*-methylpyrrolidine-2-)octanoic acid (**3a**) (80 mg, 83%); ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.20–1.40 (*m*, 18H, CH_2), 1.45–1.65 (*m*, 4H, CH_2), 1.70–1.90 (*m*, 4H, CH_2), 2.0 (*m*, 2H, C*H*, 9-*H*, 12-*H*), 2.20 (*t*, 2H, 2-*H*), 2.53 (*s*, 3H, N-CH_3), and 10.40 (*s*, 1H, COO*H*); ^{13}C NMR ($CDCl_3$, ppm): 14.06 (C-18), 22.55 (C-17), 25.93 (C-3), 26.82 (C-7), 26.97

(C-14), 27.95 (C-10, C-11), 29.25, 29.46, 29.83, 31.29 (C-8), 31.43 (C-13), 31.74 (C-16), 36.67, 37.70 (N-CH_3), 68.78 (C-9), 68.91 (C-12), and 179.29 (C-1, COOH).

(f) Hydrolysis of methyl 8-(5-hexyl-1H-pyrrole-2-)octanoate (**4**). A mixture of pyrrole ester (**4**) (0.1 g), potassium phosphate buffer (2 mL), and CCL of Lipolase (40 mg) was stirred at 40°C for 48 h. Isolation of the product gave 8-(5-hexyl-1*H*-pyrrole-2-) octanoic acid (**4a**) in 95% yield.

Chemical hydrolysis of N-*heterocyclic fatty esters: (a) Hydrolysis of methyl 8-(5-hexyl-1-pyrroline-2-yl)octanoate* (**1**). A mixture of compound **1** (0.2 g, 0.65 mmol) and ethanolic KOH (1 M, 1.2 mL) was stirred at 25°C for 2 h. The mixture was acidified with diluted HCl (6 M, 1 mL) and was extracted with dichloromethane (3 × 10 mL). The organic extract was washed with saturated aqueous NaCl (2 × 5 mL) and dried (Na_2SO_4). Evaporation of solvent gave the *N*-HCl salt of 8-(5-hexyl-1-pyrroline-2-yl)octanoic acid *N*-HCl salt (**1b**) (11 mg, 5%). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.20–1.50 (*m*, 14H, CH_2), 1.55–1.70 (*m*, 3H, CH_2, 10-*H*, N-*H*), 1.70–1.80 (*m*, 2H, ring-CH_2), 1.80–2.10 (*m*, 2H, CH_2, 8-*H*), 2.30 (*t*, 2H, 2-*H*), 2.50 (*m*, 2H, C*H*2, 14-*H*), 2.97 (*m*, 2H, CH_2, 13-*H*), 3.12 (*m*, 2H, CH_2, 11-*H*), 4.44 (*m*, 1H, C*H*, 9-*H*), and 9.46 (*s*, 1H, COO*H*); ^{13}C NMR ($CDCl_3$, ppm): 14.00 (C-18), 22.39 (C-17), 24.59 (C-3), 25.27 (C-7), 26.04 (C-14), 26.08 (C-10, ring CH_2), 28.65, 28.74, 28.93, 28.96, 32.01 (C-13), 33.92 (C-2), 34.04 (C-2), 34.12 (C-8), 36.47 (C-11, ring-CH_2), 67.22 (C-9, N*C*H), 178.18 (C-1, *C*OOH), and 195.41 (C-12, *C*=N^+).

*(b) Hydrolysis of methyl 8-(*cis/trans-*5-hexyl-pyrrolidine-2-)octanoate* (**2**). Compound **2** was hydrolyzed using ethanolic KOH by the same procedure as described above to yield *N*-HCl salt of 8-(*cis/trans*-5-hexyl-pyrrolidine-2-)octanoic acid (**2a**, 90%). ^{1}H NMR ($CDCl_3$, δ) 0.87 (*t*, CH_3, 3H), 1.20–1.1.50 (*m*, 14H, CH_2), 1.50–1.70 (*m*, 4H, CH_2), 1.70–1.90 (*m*, 2H, CH_2), 1.90–2.10 (*m*, 2H, CH_2), 2.10–2.25 (*m*, 2H, CH_2), 2.30 (*t*, 2H, CH_2), 3.48 (*m*, 2H, $^+NH_2$), 3.64 (*m*, 2H, C*H*, 9-*H*, 12-*H*, *trans*), and 10.60 (*s*, 1H, COO*H*); ^{13}C NMR ($CDCl_3$, ppm): 14.04 (C-18), 22.57 (C-17), 24.63 (C-3), 26.48 (C-7, *trans*), 26.59 (C-14, *trans*), 26.69 (C-7, *cis*), 26.83 (C-14, *trans*), 28.54, 28.57, 28.86, 28.93, 29.00, 29.03, 29.19, 30.3, 31.69 (C-16), 32.12 (C-10, *trans*), 32.24 (C-11, *trans*), 32.45 (C-10, *cis*), 32.61 (C-11, *cis*), 34.15 (C-2), 59.55 (C-9, *trans*), 59.64 (C-12, *trans*), 60.37 (C-9, *cis*), 60.46 (C-12, *cis*), and 178.42 (C-1, *C*OOH); HR–MS [*m/z* (relative intensity)]: M^+ = 297.2673 (calc. 297.2668, M^+-HCl); 154 (100), 194 (16.7), 198 (14.2), 212 (83.5), and 213 (10).

(c) Hydrolysis of methyl 8-(-cis-*5-hexyl-*N-*methyl-pyrrolidine-2-)octanoate* (**3**). Compound **3** was hydrolyzed using ethanolic KOH by the same procedure as described above to yield *N*-HCl salt of 8-(*cis*-5-hexyl-*N*-methyl-pyrrolidine-2-)-octanoic acid (**3b**, 92%). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.20–1.50 (*m*, 20H, CH_2), 1.55–1.70 (*m*, 2H, CH_2), 1.85–2.10 (*m*, 2H, CH_2), 2.15–2.25 (*m*, 2H, CH_2), 2.32 (*m*, 2H, 2-*H*), 2.82 (*s*, 3H, NCH_3), 3.05 (*s*, 1H, ^+NH) and 10.20 (*s*, 1H, COO*H*); ^{13}C NMR ($CDCl_3$, ppm): 14.03 (C-18), 22.51 (C-17), 24.71 (C-3), 26.43 (C-14), 26.62 (C-7), 27.26, 28.79, 28.82, 28.95, 29.80, 29.69, 31.61 (C-16), 34.33 (C-2), 36.87 (N-CH_3), 70.06 (C-9, C-12, 2C) and 177.78 (C-1, COO*H*). HR–MS [*m/z* (relative intensity)]: M^+ = 311.2818 (calc. 311.2824, M^+-HCl);168 (80), 226 (100), 227 (10.5).

(d) Hydrolysis of methyl 8-(5-hexyl-1H-pyrrole-2-)octanoate (**4**). Compound **4** was hydrolyzed using ethanolic KOH by the same procedure as described above to yield 8-(5-hexyl-1*H*-pyrrole-2-)octanoic acid (**4a**, 90%). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.20–1.50 (*m*, 14H, CH_2), 1.55–1.80 (*m*, 4H, CH_2), 2.30 (*t*, 2H, 2-*H*), 2.54 (*t*, 4H, CH_2, 8-*H*, 13-*H*), 5.76 (*d*, 2H, C*H*, 10-*H*, 11-*H*), 7.90 (*s*, *br*, 1H, N-*H*), and 10.40 (*s*, *COOH*); ^{13}C NMR ($CDCl_3$, ppm): 14.10 (C-18), 22.61 (C-17), 24.62 (C-3), 27.76 (C-8), 27.81 (C-13), 28.94, 29.03, 29.12, 29.15, 29.61, 29.69, 31.69 (C-16), 34.03 (C-2), 104.48 (C-10), 104.53 (C-11), 131.06 (C-9), 131.32 (C-12), and 180.10 (C-1, *C*OOH); IR (NaCl) 3200–3400 (s, N-*H*, CO*OH*), 1700 (*CO*OH), 1580 (C=C, pyrrole ring), 1420, 1200, and 770 (*CH*, pyrrole ring).

RESULTS AND DISCUSSION

The products obtained by enzymatic hydrolysis and by chemical means are summarized in Figure 1. Our initial attempts to obtain the pure fatty acids of compounds **1–3** for biological screening purposes by chemical hydrolysis of the corresponding methyl esters failed to afford the requisite free fatty acids due to the formation of the *N*-HCl salt in each case when the saponified product was acidified with dilute HCl. The HCl salt of the 1-pyrroline fatty acid was extremely water-soluble and difficult to isolate from aqueous solution by extraction with diethyl ether or dichloromethane. Repeated extraction with dichloromethane furnished only about 5% yield of the *N*-HCl salt of the 1-pyrroline fatty acid (**1a**). However, the HCl salts of compounds **2** and **3** were readily isolated by dichloromethane extraction in >90% yields. In view of the inability to produce *N*-heterocyclic fatty acids free from HCl by chemical hydrolysis, we considered enzymatic hydrolysis as a potential alternative method to the requisite free fatty acids.

Incubation of methyl 1-pyrroline ester (**1**) with bakers' yeast at 30°C for 3 d gave the corresponding 1-pyrroline fatty acid (**1a**) in 52% yield. Unhydrolyzed substrate was recovered in 38%. No reduction of the C=N bond of the 1-pyrroline system in compound **1** was observed, as the isolated product did not reveal the presence of any pyrrolidine derivatives. Reactions involving bakers' yeast involve cumbersome extraction procedures (11), which led us to concentrate on lipase-catalyzed reactions.

Incubation of methyl 1-pyrroline fatty ester (**1**) with CCL lipase in potassium phosphate buffer for 1 h at 40°C gave 52% of the corresponding fatty acid. The hydrolysis of compound **1** was complete, when the incubation period was extended to 16 h. Hydrolysis of compound **1** with Lipolase at 60°C required 36 h to complete. The reaction period using Lipolase was significantly reduced to 3 h, when the incuba-

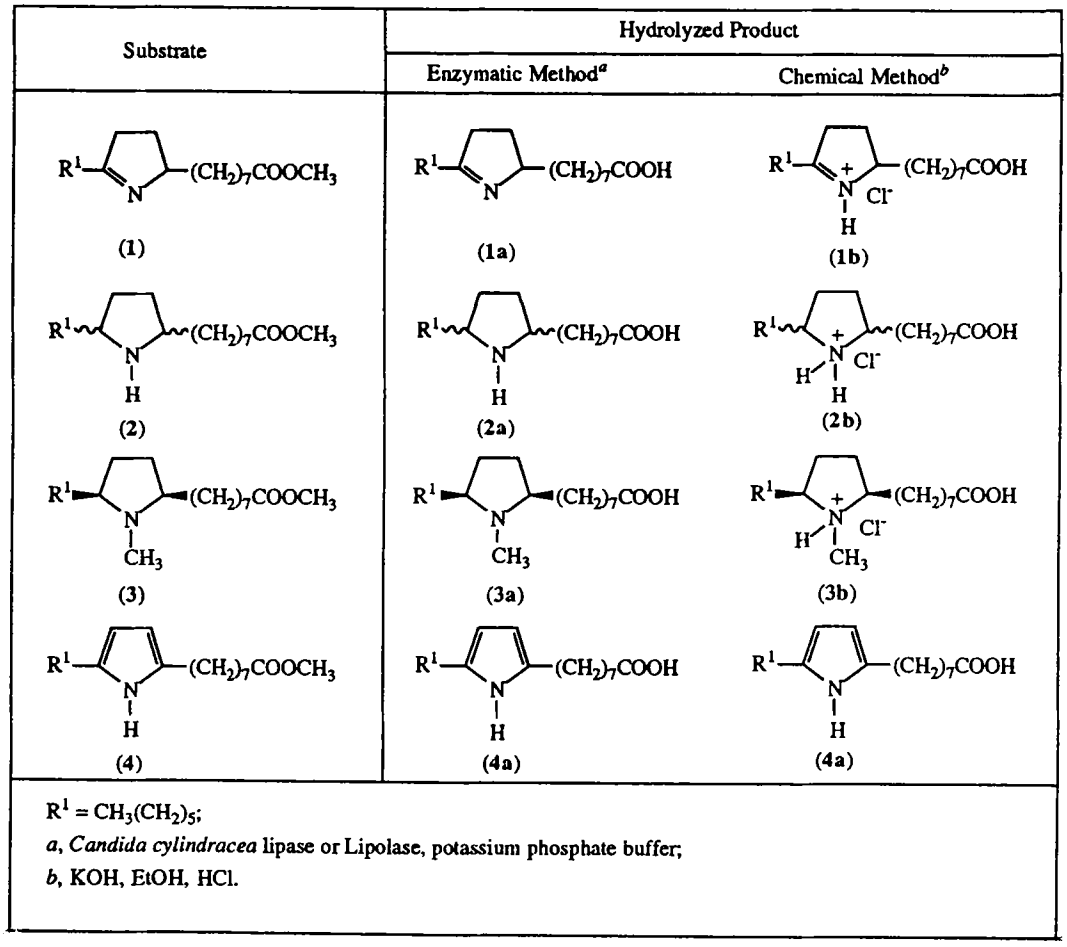


FIG. 1. Hydrolysis of *N*-heterocyclic fatty esters.

tion of compound **1** with Lipolase was carried out under ultrasound at a temperature of 57–65°C.

Attempts to hydrolyze pyrrolidine (**2**) and *N*-methyl pyrrolidine (**3**) methyl esters using CCL in potassium phosphate buffer were unsuccessful. Incubation of compound **2** for 7 d with a 2-fold amount of lipase showed no free acid product formation. From this result, it appears that the lipase of CCL is deactivated by the pyrrolidine, but not the 1-pyrroline system. Use of Lipolase to hydrolyze compound **2** at 55°C for 24 h also failed to produce the corresponding free fatty acid. However, when Lipolase-catalyzed hydrolysis of compound **2** was carried out under ultrasound at 57–65°C for 48 h, analysis of the isolated products showed the presence of the free acids (**2a**) in 51% yield, as assessed by the intensities of the carbonyl carbon signals of the ester (174.27 ppm) and acid (179.52 ppm) groups in the ^{13}C NMR spectrum of the product mixture. The hydrolysis of the *cis*-*N*-methyl pyrrolidine fatty ester (**3**) using Lipolase at 57–60°C under ultrasound condition was successful as 80% of the free acid (**3a**) was isolated after 24 h of incubation.

From these experimental results, it appears that the hydrolytic activities of CCL and Lipolase are not inhibited by the chemical nature of an *N*-heterocyclic system in the alkyl chain of the fatty esters under investigation, as the 1-pyrroline derivative is readily hydrolyzed. The resistance of the *cis*-*N*-pyrrolidine derivative to CCL and Lipolase hydrolysis reflects a stereochemical (*cis*-configuration) implication of the *N*-heterocyclic system on the enzymes, but which can be overcome by ultrasound treatment. As the 1-pyrroline system contains a planar C=N bond in its ring system, it seems that partial planarity of the ring system in the alkyl chain of a fatty ester does not cause any discriminating effects on the hydrolytic activities of such enzymes. To extend this concept to a planar *N*-heterocyclic system, we have carried out the lipase-catalyzed hydrolysis on methyl 8-(5-hexyl-1*H*-pyrrole-2-)octanoate (**4**), which contains a planar *N*-heteroaromatic system in the alkyl chain. Hydrolysis of compound **4** in the presence of CCL or Lipolase in potassium phosphate buffer at 40°C was complete in 24 h. Compound **4** was also hydrolyzed by KOH in ethanol, which on acidification with dilute HCl gave the corresponding free fatty acid (**4a**) only. No HCl-salt is formed in this case as the pyrrole ring is a heteroaromatic system.

While the saponification of methyl 1-pyrroline and pyrrolidine esters by chemical means (ethanolic KOH) is readily achieved, acidification with HCl of the saponified product

gives the *N*-HCl salts. Isolation of the *N*-HCl salt of 1-pyrroline fatty acid with dichloromethane gives a 5% yield. However, the isolation of the *N*-HCl salts of the pyrrolidine fatty acids derived from compounds **2** and **3** by solvent extraction was possible (>90% yield). The presence of chloride ions in these products was confirmed by the positive sodium fusion test (Lassaigne's test). The protonation of the nitrogen atom of the *N*-heterocyclic system caused a significant deshielding effect on the adjacent carbon atoms in the ^{13}C NMR spectra. In the case of the HCl-salt of the 1-pyrroline acid (**1b**), the protonated pyrroline nitrogen caused the carbon shift of the $C=N^+$ carbon atom to appear at 195.41 ppm, as compared to 177.07 ppm for the unprotonated 1-pyrroline carbon atom (C=N). The shifts of the methine proton (9-*H*) and carbon (9-C) nuclei of the 1-pyrroline system were also affected by the protonation of the nitrogen atom, as these signals appeared significantly more downfield than those observed for the unprotonated analogue (**1a**).

In the case of the protonated pyrrolidine (**2b**) and *N*-methyl pyrrolidine (**3b**) HCl-salts, the protonated nitrogen also caused a strong deshielding effect on the adjacent methine carbon and proton nuclei. The shifts of the *N*-methyl protons and methyl carbon atom of compound **3b** appeared at δ 2.82 and 36.87 ppm, respectively.

We conclude from this study that the hydrolytic activity of lipases from CCL and *R. miehei* (Lipolase) depends on the configuration of *N*-heterocyclic systems when present in the alkyl chain of the fatty esters. Planar and partially planar ring systems do not appear to affect the lipase activity much, but the *cis* or *trans* configuration of the ring system of 2,5-disubstituted pyrrolidines seems to cause a strong deactivation of the hydrolytic lipase activities. However, the inhibition can be overcome by conducting the reaction in ultrasound as demonstrated in these experiments.

ACKNOWLEDGMENTS

We thank the Lipid Research Fund and the Research Grants Council for financial assistance and a gift (Lipolase™) from Novo Nordisk A.S. (Hong Kong).

REFERENCES

1. Pedder, D.J., Fales, H.M., Jaouni, T., Blum, M.S., MacConnell, J., and Crewe, R.M. (1976) Constituents of the Venom of a South African Fire Ant *Solenopsis punctaticeps)* 2,5-Dialkylpyrrolidine and Pyrrolines, Identification and Synthesis, *Tetrahedron 32*, 2275–2279.
2. Jones, T.H., Blum, M.S., and Fales, H.M. (1982) Ant Venom Alkaloids from *Solenopsis* and *Monomorium* Species. Recent Developments, *Tetrahedron 38*, 1949–1958.
3. Gessner, W., Takahashi, K., Brossi, A., Kowalski, M., and Kaliner, M.A. (1987) Synthesis of (±)-*trans*-2,5-dialkylpyrrolidines from the *Lukes-Sorm.* Dilactam: Efficient Preparation of (±)-*trans*-2-butyl-5-heptylpyrrolidine and Analogs Present in Ant Venoms, *Helv. Chim. Acta 70*, 2003–2010.
4. Blum, M.S., Walker, J.R., Callahan, P.S., and Novak, A.F. (1958) Chemical, Insecticidal, and Antibiotic Properties of Fire Ant Venom, *Science 128*, 306.
5. Caro, M.R., Derbes, V.J., and Jung, R. (1957) Skin Responses to the Sting of the Imported Fire Ant *(Solenopsis saevissima)*, *Arch. Derm. 75*, 475–478.
6. Adrouny, G.A., Derbes, V.J., and Jung, R.C. (1959) Isolation of a Hemolytic Component of Fire Ant Venom, *Science 130*, 449.
7. Buffkin, D.C., and Russell, F.E. (1973) Venom of the Imported Fire Ant. Physiopharmacology, Chemistry and Therapeutics, *Proc. West. Pharmacol. Soc. 16*, 155–158.
8. Jouvenaz, D.P., Blum, M.S., and MacConnell, J.G. (1972) Antibacterial Activity of Venom Alkaloids from the Imported Fire Ant, *Solenopsis invicta*, *Antimicro. Agents Chemother. 2*, 291–293.
9. Lie Ken Jie, M.S.F., and Syed-Rahmatullah, M.S.K. (1991) Synthesis and Properties of a Novel 1-Pyrroline Fatty Ester Derivative from Methyl Isoricinoleate, *J. Chem. Soc. Perkin Trans. 1*, 421–424.
10. Lie Ken Jie, M.S.F., and Syed-Rahmatullah, M.S.K. (1991) Preparation and Some Spectroscopic Properties of *N*-Heterocyclic Derivatives of a Novel 1-Pyrroline C_{18} Fatty Ester, *Lipids 26*, 842–846.
11. Servi, S. (1990) Baker's Yeast as a Reagent in Organic Synthesis, *Synthesis*, 1–25.
12. Brockerhoff, W.H., and Jensen, R.G. (1974) *Lipolytic Enzymes*, Academic Press, New York.
13. Hoshino, T., Yamane, T., and Shimizu, S. (1990) Selective Hydrolysis of Fish Oil by Lipase to Concentrate n-3 Polyunsaturated Fatty Acids, *Agric. Biol. Chem. 54*, 1459–1467.
14. Mazur, A.W., Hiler II, G.D., Lee, S.S.C., Armstrong, M.P., and Wendel, J.D. (1991) Regio- and Stereoselective Enzymatic Esterification of Glycerol and Its Derivatives, *Chem. Phys. Lipids 60*, 189–199.
15. Lie Ken Jie, M.S.F., Sinha, S., and Ahmad, F. (1983) Chemical Transformations of C-18 Furanoid Fatty Esters, *J. Am. Oil Chem. Soc. 60*, 1777–1782.

[Received May 1, 1995, and in final revised form July 26, 1995; Revision accepted August 31, 1995]

Chemiluminescence Detection of Mono-, Bis-, and Tris-Hydroperoxy Triacylglycerols Present in Vegetable Oils

Teruo Miyazawa[a,*], Hikoichiro Kunika[a], Kenshiro Fujimoto[a], Yasushi Endo[a] and Takashi Kaneda[b]

[a]Department of Applied Biological Chemistry, Tohoku University, Tsutsumidori-Amamiyamachi, Sendai 981, Japan and [b]Koriyama Women's College, Koriyama 963, Japan

ABSTRACT: A reliable method was needed to analyze molecular species of oxidized vegetable oils. In order to accomplish this goal, mono-, bis-, and tris-hydroperoxides (Mono-OOH, Bis-OOH, and Tris-OOH, respectively) of triacylglycerols formed during autoxidation and photosensitized oxidation of oils were determined by reversed-phase high-performance liquid chromatography in combination with chemiluminescence detection (CL-HPLC). Mono-OOH was the major species (96% of total hydroperoxides) in trioleoylglycerol [peroxide value (PV) 0.16 meq/kg], and Bis-OOH and Tris-OOH showed prolonged accumulation with photooxidation. This profile was confirmed in photooxidation of trilinoleoylglycerol and trilinolenoylglycerol. Soybean oil (PV 6 meq/kg) contained Mono-OOH oleoyl-linoleoyl-linoleoylglycerol as the main peroxidic molecular species (50% of total hydroperoxides). Mono-OOH trilinoleoylglycerol was the principal species (61% of total hydroperoxides) in safflower oil (PV 5 meq/kg), and Mono-OOH oleoyl-oleoyl-linoleoylglycerol was the representative species (66% of total hydroperoxides) in olive oil (PV 3 meq/kg). The CL-HPLC method, which is specific for the detection of hydroperoxides, should prove useful in studies of triacylglycerol oxidation in foods and vegetable oils.

Lipids 30, 1001–1006 (1995).

Oxidation of food lipids and edible oils causes undesirable tastes and rancid flavors (1–5). Ingestion of rancid oils has a deleterious effect, especially on immunocompetent cells in mammals (6). Therefore, it is important to detect peroxides present in the oil.

High-performance liquid chromatography (HPLC) is one of the most effective assays in the study of triacylglycerol oxidation (7–10). The HPLC method combined with chemiluminescence detection (CL-HPLC) has been established, and this method was applied to measure phospholipid hydroperoxides (11,12), squalene hydroperoxide (13), and α-tocopherol hydroperoxide (14) in biological tissues, as well as in several food models. This CL-HPLC method has an advantage of high sensitivity as to be able to detect picomoles of hydroperoxides in each lipid class. The postcolumn-chemiluminescence reaction is specific for the hydroperoxy group, and it enables the discrimination of the hydroperoxides from the corresponding hydroxy derivatives. The application of such a highly sensitive assay to the study of oxidation in food lipids would help to understand the susceptibility of hydroperoxidation and the efficiency of antioxidants in foods and vegetable oils.

This paper describes the CL-HPLC assay of triacylglycerol hydroperoxides and its application to characterize the hydroperoxides formed during autoxidation and photosensitized oxidation of vegetable oils.

MATERIALS AND METHODS

Materials. Trioleoylglycerol, trilinoleoylglycerol (LLL), and trilinolenoylglycerol (purity above 99%) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Cytochrome *c* (from horse heart, type VI) was obtained from Sigma Chemical Co. (St. Louis, MO). Luminol (3-aminophthaloyl hydrazine), sodium borohydride, and methylene blue were from Wako Pure Chemical Co. (Osaka, Japan). *N,N*-Dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Platinum (IV) oxide hydrate was from Aldrich Chemical Co. (Milwaukee, WI). Soybean, safflower, and olive oils were purchased from a local market. All solvents and other reagents were at the highest grade available.

Photooxidation of triacylglycerols. Photosensitized oxidation of trioleoylglycerol, LLL, and trilinolenoylglycerol was performed with a slight modification of our previously report method (12). Each triacylglycerol (500 mg) was dissolved in 30 mL of chloroform/methanol (1:1, vol/vol) containing 0.1 mM methylene blue as a photosensitizer. The reaction mixture was placed in a glass beaker (200 mL) and was photoirradiated at 10°C. The light source, a 500 W photoreflector

*To whom correspondence should be addressed at Department of Applied Biological Chemistry, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Sendai 981, Japan.

Abbreviations: Bis-OOH, bis-hydroperoxides; CL-HPLC, high-performance liquid chromatography with chemiluminescence detection; DMPD, *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride; FAB–MS, fast atom bombardment–mass spectrometry; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; LLL, trilinoleoylglycerol; Mono-OOH, mono-hydroperoxides; OLL, oleoyl-linoleoyl-linoleoylglycerol; OOL, oleoyl-oleoyl-linoleoylglycerol; PLL, palmitoyl-linoleoyl-linoleoylglycerol; POL, palmitoyl-oleoyl-linoleoylglycerol; PV, peroxide value; Rt, retention time; TLC, thin-layer chromatography; Tris-OOH, tris-hydroperoxides.

Copyright © 1995 by AOCS Press

lamp (Toshiba Electronics Co., Tokyo, Japan), was held at a 50-cm distance above the surface of a reaction mixture. The photoirradiated mixture was passed through a silica column (Wako gel C-100; Wako Pure Chemical Co.) with methanol as an eluant to remove methylene blue. The methanol extract containing oxidized triacylglycerol was concentrated with a rotary evaporator, and its peroxide value (PV) was determined by potassium iodide reduction (15). The photooxidized triacylglycerol was redissolved in appropriate amounts of chloroform/methanol (2:1, vol/vol), and then analyzed by CL-HPLC with a sample injection volume of 5 to 20 μL.

Autoxidation of vegetable oils. Soybean, safflower, and olive oils were allowed to autoxidize in the dark at 25°C (6). The PV was determined by potassium iodide reduction (15). The autoxidized oil dissolved in appropriate amounts of chloroform/methanol (2:1, vol/vol) was analyzed by CL-HPLC, as in the case of photooxidized oils.

CL-HPLC. The CL-HPLC system used to determine triacylglycerol hydroperoxides was modified from that used previously (16). A reversed HPLC column, JASCO Finepak SIL C18-5 (5 μm, 250 × 4.6 mm; Japan Spectroscopic Co., Tokyo, Japan) fitted with JASCO Finepak SIL C18 T-P precolumn (5 μm, 50 × 4.6 mm), was employed. The mobile phase was methanol or a mixture of methanol/ethanol (5:1, vol/vol) and the flow rate was 1.1 mL/min. The column eluant was mixed at the post column with a chemiluminesence reagent consisting of cytochrome *c* (10 μ g/mL) and luminol (2 μ g/mL) in 50 mM borate buffer (pH 10.0). The chemiluminescence was measured with a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan). The hydroperoxide level was calibrated by known concentration of mono-, bis-, and tris-hydroperoxides (Mono-OOH, Bis-OOH, and Tris-OOH, respectively) of each triacylglycerol, except for vegetable oils, and was then expressed as picomol of hydroperoxide-0_2 (12). The concentration of triacylglycerol hydroperoxides in vegetable oils was estimated by using hydroperoxy LLL as a standard as most of the hydroperoxides formed during oxidation of vegetable oils were located on linoleic acid in triacylglycerols.

Mass spectrometry (MS). Triacylglycerol hydroperoxides isolated with preparative HPLC (Finepak SIL C18-5 and Finepak SIL C18 T-P guard column, ultraviolet detection at 210 or 234 nm) were analyzed by fast atom bombardment–MS (FAB–MS) using a JEOL JNM-HX-105 mass spectrometer (Tokyo, Japan) after reduction with sodium borohydride and hydrogenation with platinum (IV) oxide hydrate as a catalyst. The methyl esters of hydroxy fatty acids were obtained from triacylglycerol hydroperoxides after reduction, hydrogenation, and transmethylation, and then were characterized by gas–liquid chromatography (GLC) using a glass column (3 mm × 1 m) packed with Dexsil 300GC (GL Sciences, Tokyo, Japan). Constituent fatty acids of triacylglycerol molecular species separated with a reversed-phase HPLC also were determined by GLC. On the other hand, to confirm the presence of the hydroperoxy group in the molecule, oxidized oils and isolated triacylglycerol hydroperoxides were submitted to thin-layer chromatography (TLC) on a Kieselgel 60 plate (Merck, Darmstadt, Germany) with *n*-hexane/diethyl ether/acetic acid (60:40:1, by vol) as a mobile phase, and hydroperoxide detection was performed on TLC by spraying 1% DMPD in methanol solution (17).

RESULTS AND DISCUSSION

Trioleoylglycerol. Chemiluminescence chromatogram of trioleoylglycerol (PV 0.16 meq/kg) in CL-HPLC is shown in Figure 1. Two major chemiluminescence peaks of retention times (Rt) at 21.0 and 10.0 min were observed. The peak components were identified as Mono-OOH and Bis-OOH of trioleoylglycerol, respectively, from their structural characterization, as described later. Under such chromatographic conditions, unoxidized trioleoylglycerol appeared at Rt 56.0 min with ultraviolet detection at 210 nm. The chemiluminescence peaks ascribed to Mono-OOH and Bis-OOH of trioleoylglycerol completely disappeared from the chromatogram after reduction with sodium borohydride. Previously it was demonstrated that lipid hydroperoxide reduced to the corresponding hydroxy compound with sodium borohydride gave no chemiluminescence in the CL-HPLC (11,12).

Figure 2 shows the CL-HPLC chromatograms of photooxidized trioleoylglycerol. To characterize the chemiluminescence peaks, oxidized trioleoylglycerol (PV 3100 meq/kg) was analyzed by TLC, and it was confirmed to give four spots at R_f 0.57, 0.37, 0.19, and 0.13 after spraying with H_2SO_4 and heating. The three spots at R_f 0.37, 0.19, and 0.13 were identical with the compounds of chemiluminescence peaks 1, 2, and 3 in Figure 2, respectively, and the spot at R_f 0.57 was unoxidized trioleoylglycerol. Chemiluminescence com-

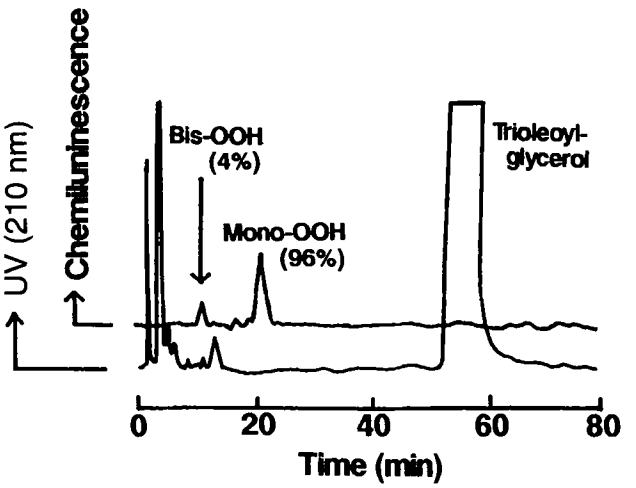


FIG. 1. High-performance liquid chromatography with chemiluminescence detection (CL-HPLC) of trioleoylglycerol (peroxide value 0.16 meq/kg). HPLC column was a Finepak SIL C18-5 (5 μm, 250 × 4.6 mm) connected with Finepak SIL C18 T-P pre-column (5 μm, 50 × 4.6 mm). The mobile phase was methanol and the flow rate was 1.1 mL/min. The chemiluminescence reagent was composed of cytochrome c and luminol. Mono-OOH, mono-hydroperoxides (retention time 21.0 min); Bis-OOH, bis-hydroperoxides (retention time 10.0 min); UV, ultraviolet.

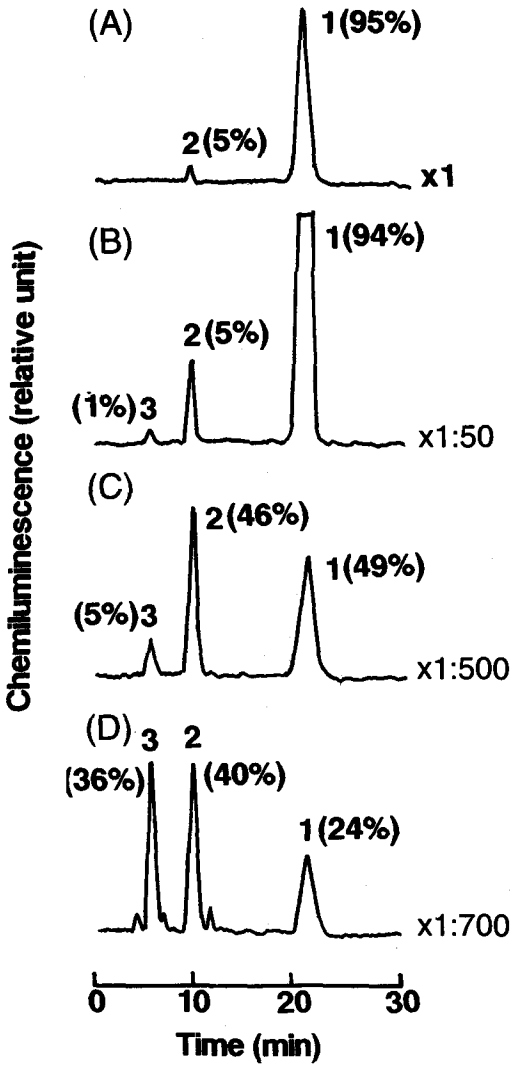


FIG. 2. CL-HPLC chromatograms of trioleoylglycerol photooxidized at 10°C for 0.5 h: A, peroxide value (PV) 4 meq/kg, 1 h; B, PV 330 meq/kg, 10 h; C, PV 3100 meq/kg and 20 h; D, PV 5000 meq/kg. Analytical conditions were the same as those given in Figure 1. Indications, ×1, ×1:50, ×1:500 and ×1:700, are the relative dilution ratios of oxidized oil. Peak 1, mono-hydroperoxides; peak 2, bis-hydroperoxides; peak 3, tris-hydroperoxides. Abbreviations as in Figure 1.

pounds of peaks 1, 2, and 3 gave a positive pink spot on a TLC plate when sprayed with DMPD reagent, which is specific for the hydroperoxy group. FAB–MS assay was conducted to further ascertain the structures. The reduced and hydrogenated compound of peak 1 component showed a molecular ion $[M + 1]^+$ at *m/z* 908 corresponding to monohydroxy tristearoylglycerol, thus indicating that the peak 1 compound was trioleoylglycerol Mono-OOH. Similarly, the compounds of peaks 2 and 3 gave $[M + 1]^+$ at *m/z* 924 and *m/z* 941, corresponding to bis- and tris-hydroxy tristearoylglycerols after reduction and hydrogenation, thus indicating that peak 2 and 3 compounds were Bis-OOH and Tris-OOH, respectively, of trioleoylglycerol.

Depending on the prolonged photooxidation periods, the PV increased, and the yield of chemiluminescence products ascribed to Mono-OOH (Rt 21.0 min), Bis-OOH (10.0 min), and Tris-OOH (6.0 min) grew (Fig. 2). As shown in Figure 3, Mono-OOH were predominant in the initial photooxidation stage of trioleoylglycerol (PV below 330 meq/kg), and a large accumulation of Bis-OOH and Tris-OOH were observed at further prolonged oxidation periods at PV above 2000 meq/kg.

LLL and trilinolenoylglycerol. The CL-HPLC chromatograms of photooxidized LLL revealed the formation of Mono-OOH, Bis-OOH, and Tris-OOH as chemiluminescence peaks (Fig. 4). The structural identification was performed in the same manner as in the case of trioleoylglycerol hydroperoxides. LLL Mono-OOH were predominant (97–98% of total hydroperoxides) at PV 120 and 480 meq/kg, although considerable concentrations of Bis-OOH and Tris-OOH appeared at PV 2400 meq/kg. In photooxidation of trilinolenoylglycerol, Mono-OOH (95% of total hydroperoxides) was a major product at PV 330 meq/kg, and Bis-OOH (17% of total hydroperoxides), but not Tris-OOH, was detected at PV 1700 meq/kg (Fig. 5). These observations were almost in agreement with those of Frankel *et al.* (8). The calibration curves of Mono-OOH formed during photooxidation of triacylglycerols were different in the chemiluminescence yield among three triacylglycerols, but a good proportional correlation ($r > 0.99$) was observed in each triacylglycerol between the accumulated hydroperoxides and the chemiluminescence yields (Fig. 6). The detection limit of LLL Mono-OOH was as low as 2 picomol under the present analytical conditions.

Vegetable oils. Figure 7 shows the CL-HPLC chromatograms of soybean oil (PV 6 meq/kg) and autoxidized soybean oil (PV 110 and 780 meq/kg). Only Mono-OOH was detected at PV 6 meq/kg, and the peroxidic molecular species con-

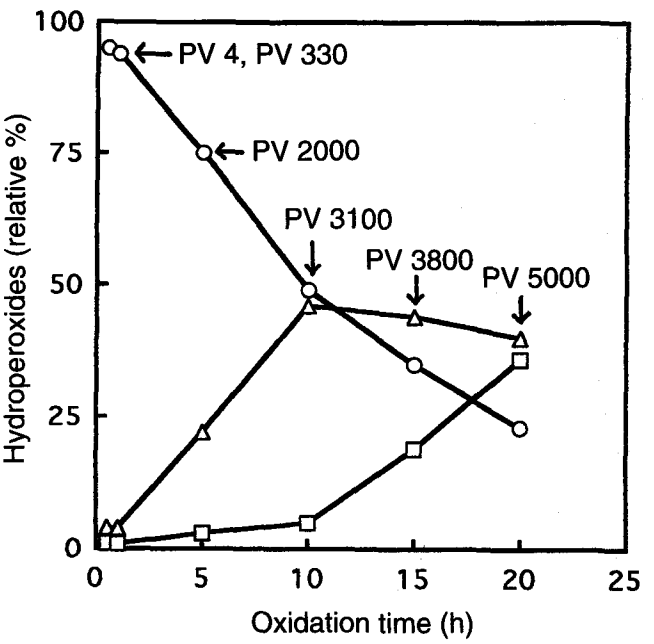


FIG. 3. Time course of mono- (○), bis- (△) and tris-hydroperoxides (□) formation during photooxidation of trioleoylglycerol as estimated by CL-HPLC. PV, peroxide value; other abbreviation as in Figure 1.

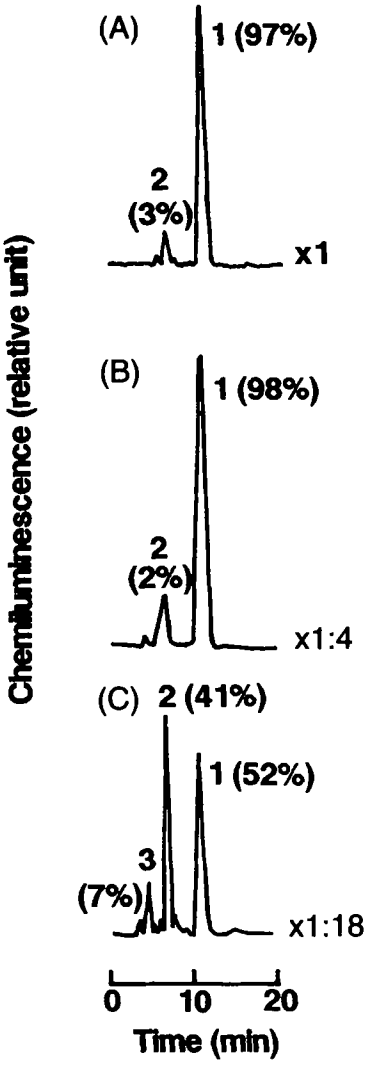


FIG. 4. CL-HPLC chromatograms of trilinoleoylglycerol photooxidized at 10°C for 5 min: A, PV 120 meq/kg, 30 min; B, PV 480 meq/kg and 5 h; C, PV 2400 meq/kg. The mobile phase was methanol/ethanol (5:1, vol/vol), and the flow rate was 1.1 mL/min. Other analytical conditions were the same as those given in Figure 1. Indications, ×1, ×1:4, and ×1:18, are the relative dilution ratios of oxidized oil. Peak 1, mono-hydroperoxides (retention time 11.0 min); peak 2, bis-hydroperoxides (6.8 min); peak 3, tris-hydroperoxides (4.8 min). Abbreviations as in Figures 1 and 3.

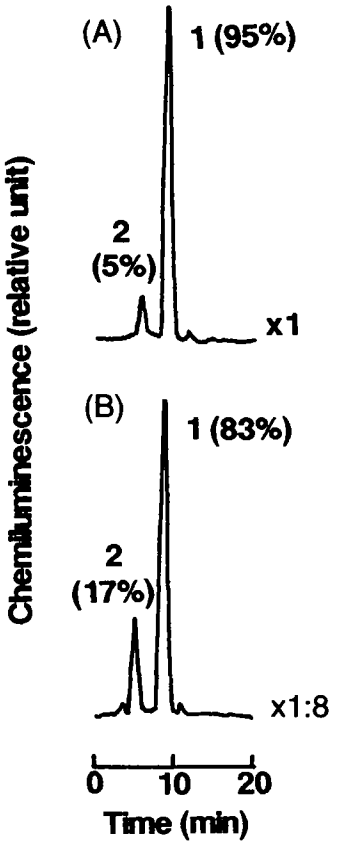


FIG. 5. CL-HPLC chromatograms of trilinolenoylglycerol photooxidized at 10°C for 5 min: A, PV 330 meq/kg and 30 min; B, PV 1700 meq/kg. Analytical conditions were the same as those given in Figure 4. Indications, ×1 and ×1:8, are the relative dilution ratios of oxidized oil. Peak 1, mono-hydroperoxides (retention time 7.9 min); peak 2, bis-hydroperoxides (5.8 min). Abbreviations as in Figures 1 and 3.

sisted of Mono-OOH of oleoyl-linoleoyl-linoleoylglycerol (OLL, 50%), LLL (28%), palmitoyl-linoleoyl-linoleoylglycerol (PLL), oleoyl-oleoyl-linoleoylglycerol (OOL, 20%), and palmitoyl-oleoyl-linoleoylglycerol (POL, 2%), which were characterized from their constituent fatty acids of the peak components analyzed by GLC. Bis-OOH at PV 110 and 780 meq/kg was 1 and 13% of the total hydroperoxides, respectively (Fig. 7). OLL Mono-OOH was the representative hydroperoxidic molecular species formed in the soybean oil autoxidation.

In safflower oil at PV 5 meq/kg, LLL Mono-OOH was predominant (61% of total hydroperoxides), followed by Mono-OOH of OLL (26%) and PLL (8%), and Bis-OOH was minor (5%), as shown in Figure 8A. In olive oil at PV 3 meq/kg, OOL Mono-OOH was the major one (66% of total hydroperoxides), followed by Mono-OOH of OLL (29%) and POL (4%). These results showed that the molecular species of Mono-OOH formed during oxidation of vegetable oils depended on their triacylglycerol composition. Bis-OOH was 1% of the total hydroperoxides in olive oil (Fig. 8B). Tris-OOH was not detected in either of the sample oils. A little quenching (negative peak) observed at Rt 7.6 min in the chemiluminescence chromatograms of safflower oil was due to α-tocopherol originally contained in the oils.

The present results demonstrate that the CL-HPLC is a useful tool for quantifying the molecular levels of hydroperoxides formed during the photooxidation and autoxidation of vegetable oils. The advantage of the CL-HPLC assay is being able to detect triacylglycerol hydroperoxides at lower concentrations such as PV 0.16–6.0 meq/kg. This high sensitivity may be very beneficial to the study of peroxidation and flavor changes in vegetable oils. A possible disadvantage is that excess amounts of α-tocopherol present in the oil might affect the chemiluminescent reaction of hydroperoxides, especially when the Rt are overlapped with each other. Fortu-

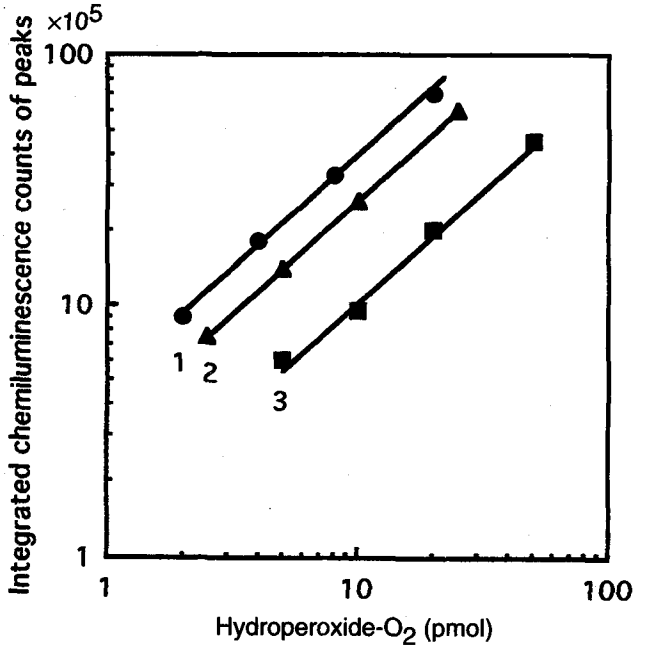


FIG. 6. Calibration curves of triacylglycerol mono-hydroperoxides as determined by CL-HPLC. Analytical conditions were the same as those given in Figure 4. 1, Trilinoleoylglycerol mono-hydroperoxides; 2, trilinolenoylglycerol mono-hydroperoxides; 3, trioleoylglycerol mono-hydroperoxides. Abbreviations as in Figure 1.

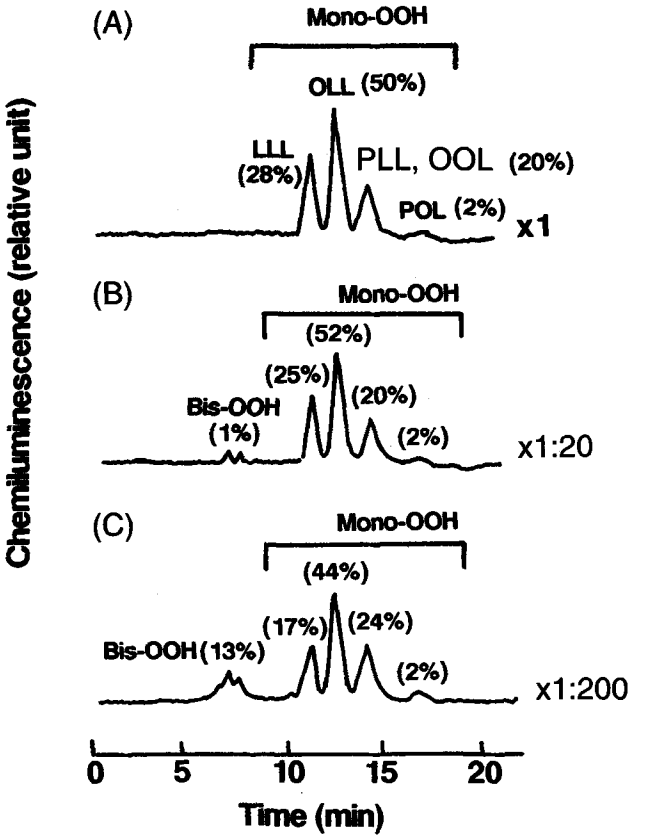


FIG. 7. CL-HPLC chromatograms of soybean oil (A, PV 6 meq/kg; B, PV 110 meq/kg; C, PV 780 meq/kg) autoxidized at 25°C. Analytical conditions were the same as those given in Figure 4. Indications, ×1, ×1:20, and ×1:200, are the relative dilution ratios of oxidized oil. LLL, OLL, PLL, OOL, and POL depicted the fatty acid combination in molecular species of triacylglycerol, with abbreviations of palmitic (P), oleic (O), and linoleic acids (L). Other abbreviations as in Figure 1.

nately, the α-tocopherol level, as generally present in commercial oils, did not cause such disturbance. The chemiluminescence reagent of a mixture of cytochrome *c* and luminol employed presently has very low reactivity to cyclic peroxides, such as epidioxides (18). Hydroperoxy cyclic peroxide has been reported to occur in oxidation of methyl linoleate (7,19) and methyl linolenate (20), but under the present analytical conditions with CL-HPLC, triacylglycerols containing such hydroperoxy cyclic peroxides were not detected at substantial levels in photooxidation of LLL and trilinolenoylglycerol. Porter *et al.* (21) reported that the rate of cyclization of initially formed peroxy radicals is in competition with H-abstraction; thus, the distribution of Mono-OOH vs. hydroperoxyepidioxide depends on the H-donating ability of the medium. Although Bis-OOH and Tris-OOH would be formed from the corresponding Mono-OOH and Bis-OOH, respectively (7,22), the principal peroxide would be Mono-OOH of triacylglycerols in vegetable oils, which are found commercially and are in PV lower than 6 meq/kg.

REFERENCES

1. Frankel, E.N. (1980) Lipid Oxidation, *Prog. Lipid Res. 19*, 1–22.
2. Frankel, E.N. (1991) Recent Advances in Lipid Oxidation, *J. Sci. Food Agric. 54*, 495–511.
3. Frankel, E.N. (1982) Volatile Lipid Oxidation Products, *Prog. Lipid Res. 22*, 1–33.
4. Grosch, V.W. (1989) Aromastoff aus der Lipidperoxidation, *Fat Sci. Technol. 91*, 1–6.
5. St. Angelo, A.J. (1992) *Lipid Oxidation in Food*, American Chemical Society, Washington, D.C.
6. Oarada, M., Miyazawa, T., Fujimoto, K., and Kaneda, T. (1989) The Effect of Dietary Autoxidized Oils on Immunocompetent Cells in Mice, *Biochim. Biophys. Acta 1012*, 156–160.
7. Neff, W.E., Frankel, E.N., Selke, E., and Weisleder, D. (1983) Photosensitized Oxidation of Methyl Linoleate Monohydroperoxides: Hydroperoxy Cyclic Peroxides, Dihydroperoxides, Keto Esters and Volatile Thermal Decomposition Products, *Lipids 18*, 868–876.
8. Frankel, E.N., Neff, W.E., and Miyashita, K. (1990) Autoxidation of Polyunsaturated Triacylglycerols. II. Trilinolenoylglycerol, *Lipids 25*, 40–47.
9. Miyashita, K., Frankel, E.N., Neff, W.E., and Awl, R.A. (1990) Autoxidation of Polyunsaturated Triacylglycerols. III. Synthetic Triacylglycerols Containing Linoleate and Linolenate, *Lipids 25*, 48–53.
10. Neff, W.E., Mounts, T.L., Rinsch, W.M., Konishi, H., and EL-Agaimy, M.A. (1994) Oxidative Stability of Purified Canola Oil Triacylglycerols with Altered Fatty Acid Composition and Structure, *J. Am. Oil Chem. Soc. 71*, 1101–1109.
11. Miyazawa, T. (1989) Determination of Phospholipid Hydroperoxides in Human Blood Plasma by A Chemiluminescence-HPLC Assay, *Free Radic. Biol. Med. 7*, 209–217.
12. Miyazawa, T., Suzuki, T., Fujimoto, K., and Yasuda, K. (1992) Chemiluminescent Simultaneous Determination of Phos-

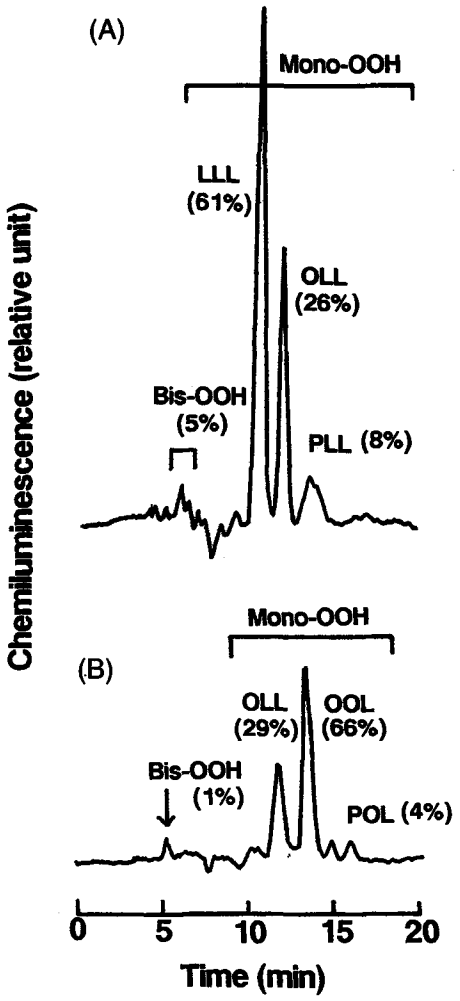


FIG. 8. CL-HPLC chromatograms of safflower oil (A, PV 5 meq/kg) and olive oil (B, PV 3 meq/kg). Analytical conditions and abbreviations of molecular species were the same as those given in Figure 7. Abbreviations as in Figures 1 and 7.

phatidylcholine Hydroperoxide and Phosphatidylethanolamine Hydroperoxide in the Liver and Brain of the Rat, *J. Lipid Res. 33*, 1151–1159.

13. Khono, Y., Sakamoto, O., Nakamura, T., and Miyazawa, T. (1993) Determination of Human Skin Surface Lipid Peroxides by Chemiluminescence-HPLC. II. Detection of Squalene Hydroperoxide, *J. Jpn. Oil Chem. Soc. 42*, 204–209.
14. Miyazawa, T., Yamashita, T., and Fujimoto, K. (1992) Chemiluminescence Detection of 8a-Hydroperoxy-Tocopherone in Photooxidized α-Tocopherol, *Lipids 27*, 289–294.
15. Asakawa, T., and Matsushita, S. (1980) A Colorimetric Microdetermination of Peroxide Values Utilizing Aluminum Chloride As the Catalyst, *Lipids 15*, 965–967.
16. Miyazawa, T., Fujimoto, K., Suzuki, T., and Yasuda, K. (1994) Determination of Phospholipid Hydroperoxides Using Luminol Chemiluminescence-High Performance Liquid Chromatography, *Methods Enzymol. 233*, 324–332.
17. Dugan, P.R., and O'Neill, R.D. (1963) Modified Spectrophotometric Reaction for Detecting Organic Peroxide, *Anal. Chem. 35*, 414–415.
18. Miyazawa, T., Fujimoto, K., and Kaneda, T. (1987) Detection of Picomole Levels in Lipid Hydroperoxides by A Chemiluminescence Assay, *Agric. Biol. Chem. 51*, 2569–2573.
19. Frankel, E.N., Neff, W.E., Selke, E., and Weisleder, D. (1982) Photosensitized Oxidation of Methyl Linoleate. Secondary and Volatile Thermal Decomposition Products, *Lipids 17*, 11–18.
20. Frankel, E.N., Neff, W.E., and Weisleder, D. (1981) High Pressure Liquid Chromatography of Autoxidized Lipids: II. Hydroperoxy-Cyclic Peroxides and Other Secondary Products from Methyl Linolenate, *Lipids 16*, 439–448.
21. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) Unified Mechanism of Polyunsaturated Fatty Acid Autoxidation. Competition of Peroxy Radical Hydrogen Atom Abstraction, β-Scission and Cyclization, *J. Am. Chem. Soc. 103*, 6447–6455.
22. Park, D.K., Terao, J., and Matsushita, S. (1981) The Isomeric Composition of Hydroperoxides Formed by Autoxidation of Unsaturated Triglycerides and Vegetable Oils, *Agric. Biol. Chem. 45*, 2071–2076.

[Received June 23, 1995, and in final revised form August 30, 1995; Revision accepted September 7, 1995]

In Vitro Oxidation of Vitamins C and E, Cholesterol, and Thiols in Rat Brain Synaptosomes

G.T. Vatassery*

Research Service and the Geriatric Research Education and Clinical Center (GRECC), VA Medical Center, Minneapolis, Minnesota 55417, and Department of Psychiatry, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT: Free radical-induced oxidation of vitamins C, E, sulfhydryl compounds, and cholesterol in brain synaptosomes from Fisher 344 rats was studied. The synaptosomes were incubated at 37°C with 2,2′-azo*bis*-(2-amidinopropane) dihydrochloride (AAPH), which undergoes thermal decomposition to yield free radicals. After incubation, the synaptosomes were sedimented, saponified, and extracted with hexane to isolate tocopherol and cholesterol. Ascorbate and tocopherol were assayed by liquid chromatography, cholesterol by gas chromatography, and total sulfhydryls by spectrophotometry. Under the *in vitro* conditions used in this study, the approximate order for the ease of oxidation of the various compounds was: ascorbate >> tocopherol > sulfhydryl compounds >>> cholesterol. However, tocopherol and sulfhydryl oxidation occurred even before all of the ascorbate had been consumed. Therefore, the fate of a specific antioxidant at a particular cellular location cannot be predicted with complete accuracy using the *in vitro* order for ease of oxidation shown here. Ascorbate may play a major role in protecting brain against oxidative damage because: (i) ascorbate concentration is high in brain, (ii) it can regenerate vitamin E from its radical oxidation product, and (iii) it is one of the first antioxidants to be consumed during oxidative reactions.
Lipids 30, 1007–1013 (1995).

Numerous studies have shown that cellular damage caused by oxidants may play a critical role in the incidence of pathological changes in a variety of diseases including cancer, heart disease, stroke, arthritis, and neurodegenerative diseases (1). Therefore, maintenance of the redox equilibrium of all compartments within a cell is important for prevention of oxidative damage in biological organisms. A number of endogenous compounds are involved in controlling the redox status of a cell. Vitamins C and E, as well as sulfhydryl groups in proteins, and low molecular weight compounds, such as glutathione, are known to play important roles in stabilizing the intra- and extracellular redox environment. As expected, biochemical and functional interactions occur among these antioxidant compounds. In 1968, Tappel (2) postulated that ascorbic acid can regenerate tocopherol from tocopheroxyl radical. The biochemical and nutritional interrelationship between vitamins C and E has been studied in detail by many investigators (see review, Ref. 3). To cite one example, using human platelets as biological cellular substrates, we have shown that ascorbic acid, glutathione, and synthetic antioxidants can prevent the oxidation of vitamin E under *in vitro* conditions (4). Thus, the functional and biochemical interrelationship between vitamins C and E have been well established.

The interactions between vitamins C and E and sulfhydryl compounds also have been studied by some investigators. Wefers and Sies (5) observed that the ascorbate-mediated protection of microsomal membranes is dependent upon the presence of vitamin E in membranes. In addition, they also found that in the presence of glutathione and ascorbate the antioxidant activity of vitamin E was maintained primarily by ascorbate. Willson (6) conducted pulse radiolysis experiments in pure chemical media and proposed that the sequence of reactions involved in the classical NADH oxidase action also may occur even when the intermediates involved are all free radicals. According to this scheme, single atoms of hydrogen are postulated to be shuttled from NADH to RS radical (derived from thiols such as glutathione) to ascorbate anion to tocopheroxyl radical to RO_2 radical (lipid peroxyl radical). This scheme suggests a functional linkage among vitamins C, E, and thiols. As far as we are aware, studies on the interrelationships between these endogenous antioxidants in subcellular fractions from mammalian brain are not available in the literature. This study was conducted to address this deficiency.

The temporal sequence of oxidation of the aforementioned antioxidants in human physiological fluids under *in vitro* conditions has been studied. Frei *et al.* (7) have reported that ascorbate prevents the peroxidation of lipids in human plasma and also spares α-tocopherol and bilirubin. When plasma was exposed to a constant flux of aqueous peroxyl radicals, the temporal order of antioxidant consumption was found to be ascorbate = plasma thiols > bilirubin > urate > α-tocopherol (7). Wayner *et al.* (8) found the relative contributions of plasma antioxidants toward the quenching of free radicals to be urate (35–65%), plasma proteins (10–50%), ascorbate (0–24%), and vitamin E (5–10%). Thus, the individual contributions toward antioxidant activity and the ease of oxida-

*Address correspondence at Research Service (151), VA Medical Center, One Veterans Drive, Minneapolis, MN 55417.

Abbreviations: AAPH, 2,2′-azo*bis*-(2-amidinopropane) dihydrochloride; BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography; TBARS, thiobarbituric acid reactive substances..

Copyright © 1995 by AOCS Press

tion of the specific antioxidants are interrelated in a complex manner. Again, data on the relative oxidizabilities of these endogenous antioxidants in brain tissue are unavailable in the literature.

It is well known that brain is absolutely dependent upon oxygen supply for normal function. Therefore, biochemical interrelationships among the antioxidants in the brain are of special importance. Despite this, little, if any, information is available in the literature on the subject. Here we present the results of a study of the rate of disappearance of antioxidants such as vitamin E, vitamin C, and sulfhydryl compounds during *in vitro* oxidation of synaptosomes by the synthetic free radical generator 2,2′ azo*bis*-(2-amidinopropane) dihydrochloride (AAPH). The latter compound releases free radicals upon thermal decomposition. The concentrations of membrane cholesterol also were determined during the oxidative reactions.

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent-grade purity from standard sources. Solvents used for chromatography were high-performance liquid chromatography (HPLC) grade from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). Other special chemicals used were purchased from the following sources: α-tocopherol, α-tocopherolquinone, and malonaldehyde tetramethyl acetal from Kodak Laboratory Chemicals (Rochester, NY); Ficoll (Type 400) from Sigma Chemical Co. (St. Louis, MO); AAPH from Polysciences, Inc. (Warrington, PA). Absolute ethanol was obtained from Midwest Solvents Company (Pekin, IL) and was redistilled prior to use. Most of the reagent-grade chemicals were from Sigma Chemical Co.

Biochemical assays. All experiments were repeated and confirmed with brain samples from separate animals on different days. Data from typical experiments are reported. The concentration of total protein was determined by the Lowry technique as modified by Markwell *et al.* (9). Lipid peroxidation *in vitro* was measured using an assay for thiobarbituric acid reactive substances (TBARS) as proposed by Asakawa and Matsushita (10). In brief, the following solutions were added to the samples: 0.1 mL $FeCl_3 6H_2O$ (0.27%, wt/vol), 1.5 mL glycine–HCl buffer (0.2 M, pH 3.6), 0.1 mL butylated hydroxytoluene (BHT) solution (0.22%, wt/vol in ethanol), 0.1 mL sodium dodecyl sulfate (5%, wt/vol), and 1.5 mL of 0.5% (wt/vol) thiobarbituric acid. The solution was mixed and then heated in a boiling water bath for 15–30 min. The sample tubes were cooled, 1 mL of glacial acetic acid and 2 mL of chloroform added, and the mixture was shaken and then centrifuged. The absorbance of the supernatant at 532 nm was measured. Standard curves for malonaldehyde were prepared using malonaldehyde tetramethyl acetal.

The concentrations of total sulfhydryl groups in the samples were determined by the method of Habeeb (11). The samples were dissolved in 0.08 M sodium phosphate buffer (pH 8.0) containing EDTA (0.5 mg/mL) and sodium dodecyl sulfate (2%) and then treated with 5,5′-dithio*bis* (2-nitrobenzoic acid). The absorbance of the solution was measured at 410 nm. Sulfhydryl concentrations were calculated using glutathione as the standard.

Determination of ascorbate by HPLC. All samples for ascorbate analyses were mixed with a solution containing metaphosphoric acid (5% final concentration, wt/vol), cysteine (0.2%, wt/vol), and EDTA (1 mM) and centrifuged at 1500 × *g* for 10 min. The supernatant containing the ascorbate was removed and kept at −70°C until analysis. Ascorbate was determined by HPLC with electrochemical detection by the method of Margolis (12). The chromatographic conditions were as follows: Aminex HPX 87H cation exchange column 300 × 7.8 mm (Bio-Rad Laboratories, Richmond, CA); eluent 5% acetonitrile in 0.1 M formic acid, flow rate 0.5 mL/min; detector Coulochem model 5100A (ESA Inc., Bedford, MA) with the D2 electrode of the 5011 analytical cell set at 0.6 V. The retention time for ascorbate was 11.2 min.

Chromatography of tocopherol, tocopherolquinone, and cholesterol. The method for determination of tocopherols and quinone by HPLC has been published (13). Briefly, 2-mL ethanol containing 0.025% (wt/vol) BHT and 0.1 mL of 30% (wt/vol) ascorbic acid were pipetted into tubes containing samples for tocopherol analyses. The mixture was saponified at 60°C for 30 min after the addition of 1 mL of 10% potassium hydroxide solution. Tubes were cooled and 2 mL of water was added followed by 2 mL of hexane containing 0.025% (wt/vol) BHT. Tocopherols and quinone were extracted into the hexane phase by vortexing for one minute. The hexane phase was separated out and evaporated under a stream of nitrogen. The residue was redissolved in mobile phase and analyzed by reverse-phase liquid chromatography using the following conditions: column, ultrasphere ODS, 5 microns, 4.6 × 150 mm (Beckman Instruments, Fullerton, CA); mobile phase, methanol/water (94.5:5.5, vol/vol), with 7.5 mM sodium dihydrogen phosphate (final concentration); flow rate, 2.7 mL/min. The tocopherols and tocopherolquinone were detected electrochemically using a Coulochem 5100 A instrument (ESA Inc.) equipped with 5011 analytical cell with detector 1 at a potential of −0.25 V and detector 2 at +0.55 V and 5021 conditioning cell at −0.75 V. The retention times for the various compounds were: tocol (internal standard), 4.2 min; α-tocopherolquinone, 5.2 min; and α-tocopherol 8.5 min.

One portion of the hexane extract was used for cholesterol determination. After evaporation of the solvent, trimethylsilyl ether derivatives of cholesterol were prepared using *bis*(trimethylsilyl)trifluoroacetamide and analyzed by a Hewlett-Packard (Avondale, PA) model 5880 gas chromatograph equipped with a flame-ionization detector and a capillary column (DB1 column, 30 m × 0.324 mm, film thickness 0.1 μm; temperature programming conditions: oven temperature initial 50°C, initial time 3 min, program rate 30°C per min, final value 230°C, second program rate 4°C per min, final temperature 310°C and kept at 310°C for 3 min). Under these conditions, the retention times of the internal standard cholestane and cholesterol were 17.43 and 21.40 min, respectively.

Preparation of subcellular fractions from rat brain. Four-month-old, male Fisher 344 rats (Harlan Sprague Dawley, Indianapolis, IN) were used. The subcellular fractions were isolated from cerebral hemispheres by standard centrifugation methods (14). The Ficoll solution used was purified by dialyzing a 40% (wt/vol) aqueous solution against water for three hours. The final Ficoll concentration was estimated by using a graph relating density and concentration. Brain tissue was homogenized in 10 vol of ice-cold isolation medium containing 0.32 M sucrose, 10 mM HEPES, and 1mM EDTA at pH 7.4, using a glass-glass homogenizer. The homogenate was centrifuged at 1300 × *g* for 3 min and the supernatant saved. The pellet was resuspended in 10 mL of the isolation medium, rehomogenized, and centrifuged at 1300 × *g* for 3 min. The pooled supernatants were centrifuged at 17,000 × *g* for 10 min to get the crude mitochondrial fraction. The resulting pellet was resuspended in 15 mL of isolation medium. Half of this suspension was layered over 11 mL of 7.5% (wt/vol) Ficoll medium, which had been layered over 11 mL of 10% (wt/vol) Ficoll medium. The tubes were centrifuged in a Beckman SW 28 rotor at 99,000 × *g* for 45 min. The fraction at the interface between the two Ficoll solutions was removed, diluted 1:5 (vol/vol) with isolation medium, and centrifuged for 10 min at 17,000 × *g* to isolate the synaptosomes. The purity of the synaptosomal fraction was tested by electron microscopy and also by estimating the activity of the marker enzyme sodium potassium ATPase (15). The activity of the ATPase was usually enriched threefold in synaptosomes compared with the crude homogenate.

Protocol for incubations. Synaptosomes were incubated in a medium (pH 7.4) simulating extracellular fluids or plasma and contained 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, and 10 mM glucose. HEPES (4 mM) also was added for additional buffering capacity. After incubation, the tubes were placed on ice to stop the generation of free radicals. The reaction mixture was then centrifuged for 30 min at 17,000 rpm in a Sorvall SS 34 rotor (35,000 × *g*) to sediment the synaptosomes. The sedimented fractions were then analyzed for various components. In order to test whether the synaptosomal structural integrity is retained during incubations in the presence of AAPH, we estimated the release of lactate dehydrogenase into the medium using the method of Clark and Lai (16). Even when incubated in the presence of 10 mM AAPH for two hours, we could not detect any lactate dehydrogenase in the medium. Nearly all of the activity was accounted for in the pellet, indicating that no activity was lost during incubations.

A brief description of the experimental strategy follows: In every experiment a control sample was mixed with the medium and kept cold. The mixture was centrifuged and analyzed for the various components and the concentrations were used as the original basal levels for each component. The experimental and control tubes were incubated for varying lengths of time. After the lapse of specific times, the experimental and control tubes were removed and processed.

RESULTS

It was mentioned in the Materials and Methods section that incubations with AAPH did not cause release of lactate dehydrogenase into the medium. In addition, we also have found that only small amounts of protein and cholesterol were released into the medium during incubations with AAPH. The amounts released during incubations in the presence of AAPH were not significantly different from control tubes. Tocopherols, sulfhydryls, and ascorbate released into the medium were oxidized by AAPH if the latter was present in the medium. Therefore, it would not be possible to determine whether AAPH treatment results in the release of these compounds into the medium. The situation with ascorbate was even more complicated. Significant and measurable amounts of ascorbate were released into the medium when control synaptosomes were incubated without AAPH. The ascorbate released into the medium always was assayed separately. The total ascorbate was then calculated and the results used in the figures. The small amounts of tocopherol, protein, and cholesterol that were found in the supernatant could be due to the presence of fragments of membranes which were sucked up during the collection of the supernatants using pasteur pipettes. In any case, the observations that additional protein, lactate dehydrogenase, and cholesterol were not released into the medium when synaptosomes were incubated with AAPH suggest that treatment with AAPH did not result in any significant rupturing of synaptosomes.

The normal concentrations of ascorbate, α-tocopherol, total thiols, and cholesterol, as well as α-tocopherolquinone in synaptosomes are given in Table 1. It can be seen that there are substantial differences in the steady-state concentrations of the various antioxidants in the synaptosomes.

Among the different compounds studied, ascorbate and sulfhydryl compounds can be characterized as water-soluble antioxidants. The oxidations of ascorbate and total sulfhydryls in synaptosomal samples incubated in the presence or absence of 2 mM AAPH are shown in Figure 1. The oxidizability of ascorbate was considerably more than that of sulfhydryl compounds. In fact, a substantial percentage of ascorbate was oxidized even during incubations in the absence of AAPH. The oxidations of two lipid-soluble components, α-tocopherol and cholesterol, were compared. The re-

TABLE 1
Concentrations of Ascorbic Acid, Total Thiols, α-Tocopherol, and Cholesterol in Rat Brain Synaptosomes[a]

Component	Concentration (nmol/mg protein)
Ascorbic acid	5.5 ± 0.3[b]
Total thiols	92 ± 3
α-Tocopherol	0.43 ± 0.01
α-Tocopheroluinone	0.0035 ± 0.003
Cholesterol	377 ± 3

[a]Baseline levels of the different compounds were determined in rat brain synaptosomes isolated by centrifugation techniques.
[b]Mean ± SD (n = 3).

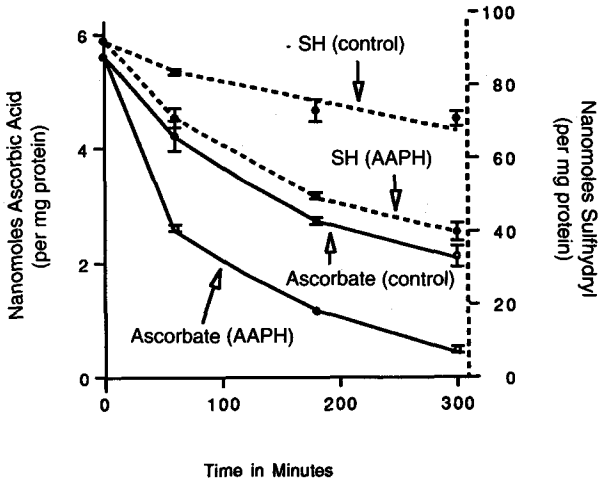


FIG. 1. Oxidation of ascorbic acid and total sulfhydryl compounds in rat brain synaptosomes induced by incubation at 37°C with the free radical generator (2,2′-azo*bis*-(2-amidinopropane) dihydrochloride (AAPH). Synaptosomes were isolated from rat cerebral hemispheres and incubated in isotonic media with 2 mM AAPH, and then ascorbate and sulfhydryl compounds were estimated. The individual incubation tubes contained 500 μg of total protein. Each point represents mean ± SD from quadruplicate assays. SH, thiols.

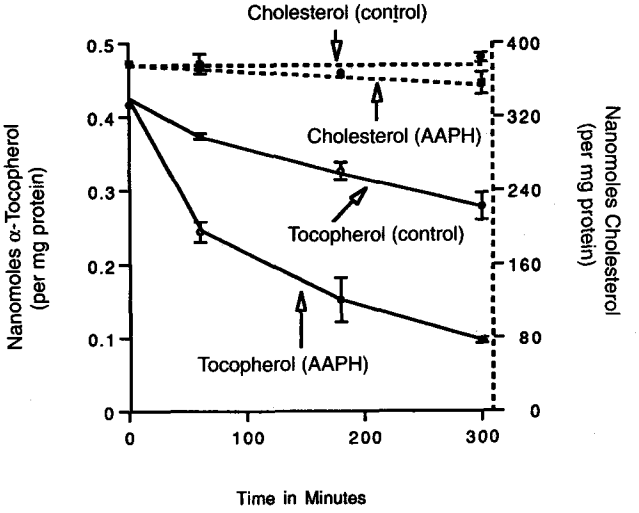


FIG. 2. Oxidation of α-tocopherol and cholesterol in rat brain synaptosomes induced by incubating at 37°C with the free radical generator AAPH. Synaptosomes were isolated from rat cerebral hemispheres and incubated in isotonic media with 2 mM AAPH, and then α-tocopherol and cholesterol concentrations were estimated. The individual incubation tubes contained 500 μg of total protein. Each point represents mean ± SD from quadruplicate assays. Abbreviations as in Figure 1.

sults (Fig. 2) show that cholesterol was very resistant to oxidation.

It can be seen from Figures 1 and 2 that the rates of oxidation of α-tocopherol and sulfhydryl compounds are quite similar. Therefore, the relationship between the rates of oxidation of these two components was studied in a single, separate experiment and the results are shown in Figure 3. The data show that when compared with oxidation of sulfhydryl compounds, a higher percentage of α-tocopherol tends to be oxidized.

Oxidations of the various compounds were followed in all experiments discussed here by determining the declines in concentration of the respective original compound. Tocopherolquinone is produced during the oxidation of tocopherol, and its rate of production was determined. The results (Fig. 4) show that production of α-tocopherolquinone paralleled the oxidation of α-tocopherol. Only a relatively small percent of the α-tocopherol oxidized was converted to tocopherolquinone. For example, at three hours of incubation, the conversion rate was 27.4 and 23% for control and AAPH-treated samples, respectively. The low yields of quinone must be due to the reaction of the tocopheroxyl radical with other substances.

Production of malondialdehyde as determined by the TBARS method is often used as a measure of lipid peroxidation. Even though this method is not very specific under *in vivo* conditions, TBARS do provide useful data on experimental induction of lipid peroxidation under *in vitro* conditions. The results of the measurements of TBARS during oxidation (Fig. 5) show that AAPH treatment is inducing lipid peroxidation. Thus, it can be concluded that incubations of

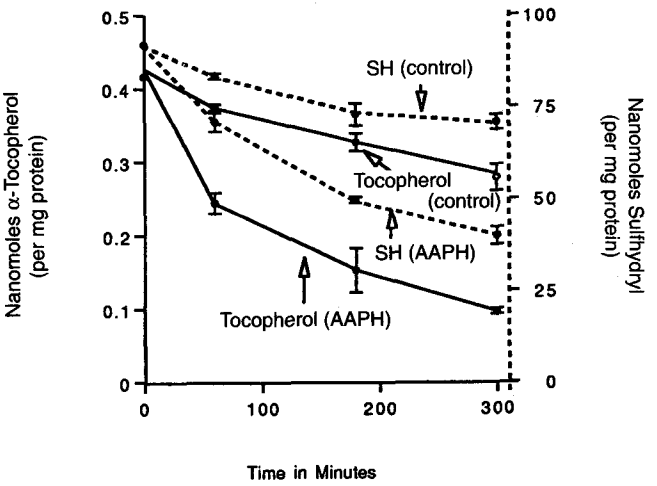


FIG. 3. Oxidation of α-tocopherol and total sulfhydryl compounds in rat brain synaptosomes. The oxidation was induced by incubating synaptosomes from rat cerebral hemispheres in isotonic media with the free radical generator AAPH (2 mM) at 37°C, and then ascorbate and sulfhydryl compounds were estimated. The individual incubation tubes contained 500 mg of total protein. Each point represents mean ± SD from quadruplicate assays. Abbreviations as in Figure 1.

synaptosomes with AAPH induce oxidations for the following reasons: (i) antioxidants such as ascorbate and tocopherol are consumed; (ii) tocopherolquinone, an oxidation product of tocopherol, is formed; and (iii) TBARS are formed.

The concentration of the oxidizing agent AAPH was 2 mM in all of the experiments described previously. The pattern of results obtained with 20 mM is nearly the same. However, if a higher concentration of AAPH (20 mM) is used as an oxi-

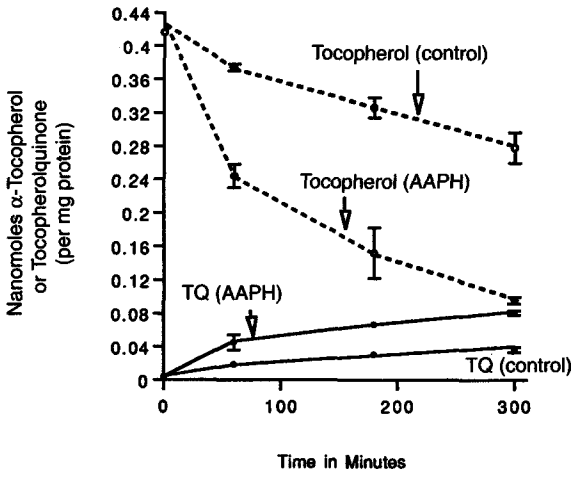


FIG. 4. Production of tocopherolquinone from synaptosomes upon incubations at 37°C with the free radical generator, AAPH. Synaptosomes were isolated from rat cerebral hemispheres and incubated in isotonic media with 2 mM AAPH, and then tocopherolquinone was estimated. The individual incubation tubes contained 500 μg of total protein. Each point represents mean ± SD from quadruplicate assays. Abbreviations as in Figure 1; *TQ, tocopherolquinone.*

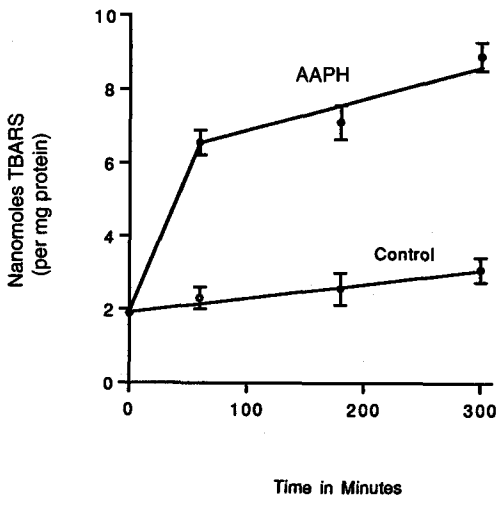


FIG. 5. Production of thiobarbituric acid reactive substances (TBARS) from synaptosomes upon incubations at 37°C with the free radical generator AAPH (2 mM). Synaptosomes were isolated from rat cerebral hemispheres and incubated for varying lengths of time. TBARS were estimated spectrophotometrically. The individual incubation tubes contained 500 mg of total protein. Each point represents mean ± SD from quadruplicate assays. Abbreviations as in Figure 1.

dant, cholesterol is oxidized (Fig. 6). As most of the α-tocopherol in the synaptosomes is oxidized before any of the cholesterol, it can be concluded that vitamin E is performing its role as a lipid-soluble antioxidant in preventing oxidation of cholesterol.

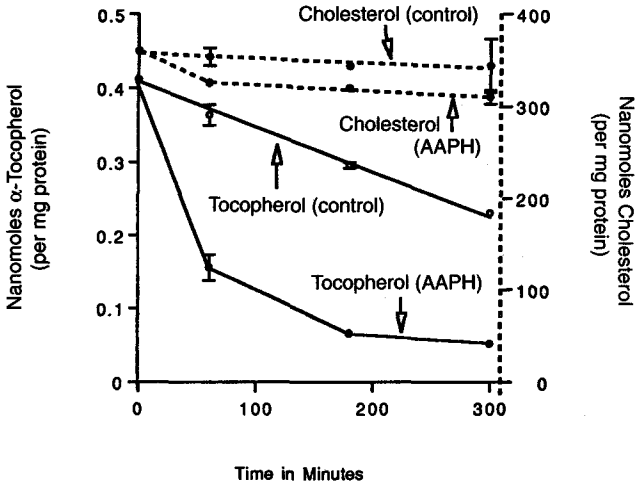


FIG. 6. Oxidations of cholesterol and α-tocopherol in rat brain synaptosomes incubated at 37°C with higher concentrations (20 mM) of the free radical generator, AAPH. Synaptosomes were isolated from rat cerebral hemispheres and incubated in isotonic media with AAPH, and then cholesterol and α-tocopherol were estimated. The individual incubation tubes contained 500 mg of total protein. Each point represents mean ± SD from quadruplicate assays.

DISCUSSION

Some investigators have suggested that compounds such as ascorbate, tocopherol, and thiols are consumed in a specific sequential order when a biological substrate like plasma is oxidized (7) . Even though compounds such as ascorbic acid are much more easily oxidized than sulfhydryl compounds, we did not find a strict sequential order of oxidation of various compounds. For example, tocopherol is being oxidized even when there is ascorbic acid remaining in the fraction. Buettner (17) has reported oxidation–reduction potentials for free radical redox couples derived from endogenous redox-active biological compounds. He has proposed that these redox potentials can be used like the classical listing of redox potentials for compounds in mitochondria, and that their order is based on the one-electron reduction potentials of the various species. Such thermodynamic tables of reactivity are indeed quite useful in predicting and understanding the behavior of the various components in biological systems. However, additional data from experiments with cells or subcellular organelles also are needed to assess the *in vivo* oxidizability of various compounds. Furthermore, as Buettner has pointed out, the redox sequence may also be changed by alterations of equilibria by various events. For example, changes in concentrations of the oxidized or reduced species result not only from removal of a component by diffusion or binding but also from enzymatic processes which replenish or utilize the component. Ultimately all of these factors are capable of changing the order of oxidation predicted solely on the basis of thermodynamic considerations. Nevertheless, a rough guide on the relative order of oxidizability of the various species can be obtained from studies using biological matrices, such as subcellular fractions.

As expected, the steady-state levels of the various compounds in synaptosomes are distinctly different from one another (Table 1). The very low levels of α-tocopherolquinone compared with that of α-tocopherol suggest that synaptosomes were not subjected to oxidation during the isolation procedure. The concentration of total thiols is much higher than those of tocopherol or ascorbate. The majority of total thiols (about 75%) are in proteins (data not shown). It is unknown how much of the protein-bound or unbound thiols are available for antioxidant protection. In addition to the steady-state concentrations of various antioxidants, the intracellular site of oxidation will play an important role in determining which antioxidant is of functional significance at a particular subcellular site.

A report dealing with a comparison of the rates of oxidation of vitamin E, protein sulfhydryls, and production of lipid peroxidation in rat liver microsomes was published by Scheschonka *et al.* (18). One part of their study involved oxidation of microsomes with AAPH. It is interesting to compare the results of Scheschonka *et al.* (18) with our data. In agreement with our data, they also have found that lipid peroxidation was preceded by the loss of most of the α-tocopherol. Their observation that α-tocopherol tended to get oxidized slightly faster than protein sulfhydryls is similar to ours. However, the ease of oxidation of these compounds in human plasma (7) is different from our observations, as well as those of Scheschonka *et al.* (18). One reason for this difference could be that plasma is much more homogeneous than subcellular fractions. In addition, antioxidants occur in specific compartments within the cell or subcellular fractions such as synaptosomes. Furthermore, mechanisms for the regeneration of antioxidants are available in the subcellular fractions and these are also compartmentalized. All of these factors would tend to make the utilization of antioxidants in plasma quite different from those of cells or subcellular structures.

The results from this study show that ascorbate is one of the first antioxidants undergoing substantial oxidation during oxidative stress of synaptosomes (Fig. 1). It is well known that among mammalian organs the brain contains one of the highest concentrations of ascorbic acid (19). Ascorbate is transported actively from blood to cerebrospinal fluid and into brain cells (20). This active transport system maintains the concentration of ascorbate in spinal fluid at a level four times that in plasma (20). In addition to being an antioxidant, ascorbate may serve other functions as well. Ascorbate is known to be a cofactor for several mono- and dioxygenases, and its mode of action is dependent on its ability to keep the metal cofactors, iron and copper, in the required reduced state (21). Thus, the reducing power of ascorbate is intimately involved with its mechanism of action as a cofactor. Ascorbate may play a major role in antioxidant protection of brain for at least three reasons. First, as mentioned previously, ascorbate concentrations are high in brain. Second, ascorbate can regenerate vitamin E, the major chain-breaking lipid-soluble antioxidant in biological membranes (2,3). Third, as shown by our data, ascorbate is one of the first components to be oxidized during challenge from an oxidant.

The interactions between thiols such as glutathione and vitamin E have been studied by a number of investigators. Tirmenstein and Reed (22) found that addition of glutathione to the incubation medium lowered the threshold level of α-tocopherol that was required for inhibition of lipid peroxidation. Studies of injury to hepatocytes caused by the chemicals adriamycin and ethacrynic acid have demonstrated that vitamin E protects against cellular damage by helping to maintain adequate levels of protein thiols (23). In experiments with homogeneous solutions, Niki *et al.* (24) found that glutathione can regenerate vitamin E from α-chromanoxyl radical. However, direct demonstrations of the reduction of tocopheroxyl radical by glutathione or other sulfhydryl compounds in cells or subcellular organelles have not been achieved so far. One report in the literature indicates that the presence of glutathione is associated with lower concentrations of tocopheroxyl radicals at steady state (25). Murphy *et al.* (26) found that protection of protein thiols was diminished in rat liver microsomes lacking vitamin E. Our data show that the oxidation of vitamin E is slightly faster than that of sulfhydryl compounds in synaptosomal fractions. This observation would be consistent with a protective role for vitamin E in preventing sulfhydryl oxidation.

One of the interesting findings in this study is that ascorbate which is confined to the cytoplasmic compartment of the synaptosome is utilized at a rapid rate in synaptosomes exposed to AAPH. This oxidation is induced by adding the water-soluble initiator AAPH into incubation medium. As significant amounts of ascorbate appear in the supernatant during incubation of synaptosomes without AAPH (see the Results section), it is possible that AAPH is oxidizing the ascorbate that is released into the medium. Other possibilities are that free radicals from AAPH cross the plasma membranes to initiate oxidation of ascorbate within the cytoplasm or radicals from AAPH induce an oxidative process in the membrane that is then transmitted to the cytoplasmic compartment within the synaptosomes. Which one of these processes is responsible for the oxidation of ascorbate cannot be decided using data from experiments in this report.

In conclusion, the results of our experiments show that an approximate order of the ease of oxidation of the various compounds within the synaptosomes is as follows: ascorbate >>α-tocopherol>sulfhydryls>>>cholesterol. It should be emphasized that smaller amounts of a component like α-tocopherol can be oxidized even when significant amounts of the more oxidizable ascorbate are still present in the synaptosome. Changes in concentrations of the various antioxidant components *via* removal by diffusion or compartmentation or replenishment by enzymatic means could substantially alter the observed order of oxidation under *in vivo* or *in vitro* conditions. Ascorbate may be one of the first antioxidants involved in the defense against oxidative damage within nerve cells, and α-tocopherol might be playing specific roles within the lipid bilayers of membranes. While assessing the antioxidant protection of the neuron, it is essential to examine the individual contributions as well as the interactions among the

various individual components of the antioxidant protective mechanisms.

ACKNOWLEDGMENTS

This study was supported by research funds from the Department of Veterans Affairs, Washington D.C., and from the National Institutes of Aging grant number RO1 AG 10528. The author wishes to thank W. Ed Smith and Hung T. Quach for expert technical assistance, and Dr. J.C.K. Lai for many helpful discussions.

REFERENCES

1. Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
2. Tappel, A.L. (1968) Will Antioxidant Nutrients Slow Aging Process? *Geriatrics 23*, 97–105.
3. McCay, P.B. (1985) Vitamin E: Interactions with Free Radicals and Ascorbate, *Ann. Rev. Nutr. 5*, 323–340.
4. Vatassery, G.T., Smith, W.E. , and Quach, H.T. (1989) Ascorbic Acid, Glutathione and Synthetic Antioxidants Prevent the Oxidation of Vitamin E in Platelets, *Lipids 24*, 1043–1047.
5. Wefers, G., and Sies, H. (1988) The Protection by Ascorbic Acid and Glutathione Against Microsomal Lipid Peroxidation is Dependent on Vitamin E, *Eur. J. Biochem. 174*, 353–357.
6. Willson, R.L. (1983) Free Radical Protection: Why Vitamin E, Not Vitamin C, Beta Carotene or Glutathione, in *Biology of Vitamin E*, Ciba Foundation Symposium, Vol. 101, pp 19–37, The Pitman Press, London.
7. Frei, R.B., England, L., and Ames, B.N. (1989) Ascorbate Is an Outstanding Antioxidant in Human Blood Plasma, *Proc. Natl. Acad. Sci. USA. 86*, 6377–6381.
8. Wayner, D.D.M., Burton, G.W., Ingold, K.U., Barkley, L.R.C., and Locke, S.J. (1987) The Relative Contributions of Vitamin E, Urate, Ascorbate and Proteins to the Total Peroxyl-Radical Trapping Activity of Human Blood Plasma, *Biochim. Biophys. Acta 924*, 408–419.
9. Markwell, M.A.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978) A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples, *Anal. Biochem. 87*, 206–210.
10. Asakawa, T., and Matsushita, S. (1980) Coloring Conditions of Thiobarbituric Acid for Detecting Lipid Hydroperoxides, *Lipids 15*, 137–140.
11. Habeeb, A.F.S.A. (1972) Reactions of Protein Sulfhydryl Groups with Ellman's Reagent, *Methods in Enzymology XXV*, 457–464.
12. Margolis, S.A. (1988) Stabilization of Ascorbic Acid in Plasma, and Its Liquid Chromatographic Determination, *Clin. Chem. 34*, 2217–2223.
13. Vatassery, G.T., Smith, W.E., and Quach, H.T. (1993) A Liquid Chromatographic Method for the Simultaneous Determination of Alpha Tocopherol and Tocopherolquinone in Human Red Blood Cells and Other Biological Samples Where Tocopherol Is Easily Oxidized During Sample Treatment, *Anal. Biochem. 214*, 426–430.
14. Lai, J.C.K., and Clark, J.B. (1989) Isolation and Characterization of Synaptic and Nonsynaptic Mitochondria from Mammalian Brain, in *Neuromethods, Volume 11: Carbohydrates and Energy Metabolism* (Boulton, A.A., and Baker, G.B., eds.) pp. 43–97, Humana Press, Clifton.
15. Maynard, R.R., Fullerton, D.S., and Ahmed, K.A. (1982) A Simple Method for the Purification of Rat Brain Na, K Adenosine Triphosphatase, *J. Pharmacol. Methods 7*, 279–288.
16. Clark, J.B., and Lai, J.C.K. (1989) Glycolytic, Tricarboxylic Acid Cycle and Related Enzymes in Brain, in *Neuromethods, Volume 11: Carbohydrates and Energy Metabolism* (Boulton, A.A., and Baker, G.B., eds.) The Humana Press, Inc., Clifton, pp. 233–281.
17. Buettner, G.R. (1993) The Pecking Order of Free Radicals and Antioxidants: Lipid Peroxidation, Alpha Tocopherol and Ascorbate, *Arch. Biochem. Biophys. 300*, 535–543.
18. Scheschonka, A., Murphy, M.A., and Sies, H. (1990) Temporal Relationships Between the Loss of Vitamin E, Protein Sulfhydryls and Lipid Peroxidation in Microsomes Challenged with Different Prooxidants, *Chem. Biol. Interact. 74*, 233–252.
19. Rajalakshmi, R., Malathy, J., and Ramakrishnan, C.V. (1967) Effect of Dietary Protein Content on Regional Distribution of Ascorbic Acid in Rat Brain, *J. Neurochem. 14*, 161–167.
20. Spector, R. (1989) Micronutrient Homeostasis in Mammalian Brain and Cerebrospinal Fluid, *J. Neurochem. 53*, 1667–1674.
21. Padh, H. (1991) Vitamin C: New Insights into Its Biochemical Functions, *Nutr. Rev. 49*, 65–70.
22. Tirmenstein, M., and Reed, D.J. (1989) Effect of Glutathione on the Alpha Tocopherol-Dependent Inhibition of Nuclear Lipid Peroxidation, *J. Lipid Res. 30*, 959–965.
23. Pascoe, G.A., Olafsdottir, K., and Reed, D.J. (1987) Vitamin E Protection Against Chemical-Induced Cell Injury. I. Maintenance of Cellular Protein Thiols as a Cytoprotective Mechanism, *Arch. Biochem. Biophys. 256*, 150–158.
24. Niki, E., Tsuchiya, J., Tanimura, R., and Kamiya, Y. (1982) Regeneration of Vitamin E from Alpha Chromanoxyl Radical by Glutathione and Vitamin C, *Chem. Lett. Chem. Soc. Japan*, 789–792.
25. Packer, L., Maguier, J.J., Mehlhorn, R.J., Serbinova, E., and Kagen, V.E. (1989) Mitochondria and Microsomal Membranes Have a Free Radical Reductase Activity That Prevents Chromanoxyl Radical Accumulation, *Biochem. Biophys. Res. Commun. 159*, 229–235.
26. Murphy, M.E., Scholich, H., and Sies, H. (1992) Protection by Glutathione and Other Thiol Compounds Against the Loss of Protein Thiols and Tocopherol Homologs During Microsomal Lipid Peroxidation, *Eur. J. Biochem. 210*, 139–146.

[Received January 17, 1994, and in final revised form July 17, 1995; Revision accepted July 24, 1995]

Biodiscrimination of α-Tocopherol Stereoisomers During Intestinal Absorption

Chikako Kiyose[a,*], Riho Muramatsu[a], Yoko Fujiyama-Fujiwara[a], Tadahiko Ueda[b] and Osamu Igarashi[a]

[a]Institute of Environmental Science for Human Life, Ochanomizu University, Bukyo-ku, Tokyo 112, Japan and [b]Tokyo Metropolitan Research Laboratory of Public Health, Shijuku-ku Tokyo 169, Japan

ABSTRACT: Synthetic α-tocopherol (α-Toc) contains equal amounts of eight different stereoisomers, and the four stereoisomers with the 2*R* configuration are generally more active than their corresponding 2*S*-isomers. We investigated the biodiscrimination of α-Toc stereoisomers during intestinal absorption *in situ* and *in vitro*. Intestinal absorption of α-Toc stereoisomers was examined *in situ* in vitamin E-deficient rats with cannulated thoracic ducts. We found that the ratios of α-Toc stereoisomers in lymph of the all-*rac*-α-Toc group were the same as the administered α-Toc stereoisomers, and 2*R*-isomers occupied approximately 50% of absorbed α-Toc. The uptake of α-Toc stereoisomers also was measured using Caco-2 cells cultured on filter membranes. The concentration of *RRR*-α-Toc in Caco-2 cells was not significantly different from that of *SRR*-α-Toc. Therefore, the discrimination of α-Toc stereoisomers does not occur during absorption in small intestine, suggesting the liver as a source for the biodiscrimination.
Lipids 30, 1015–1018 (1995).

Synthetic α-tocopherol (α-Toc) contains equal amounts of eight different stereoisomers of α-Toc arising from three asymmetric carbons (2-position in the chroman ring and 4′- and 8′-position in side chain tail). Biological activities of all the stereoisomers of α-Toc were determined by rat resorption–gestation test as follows: *RRR* = 100%, *RRS* = 90%, *RSS* = 73%, *SSS* = 60%, *RSR* = 57%, *SRS* = 37%, *SRR* = 31%, *SSR* = 21% (1). This difference in biological activities in all the stereoisomers would suggest that their discrimination occurs in the absorption, uptake, and metabolism of each stereoisomer.

In 1987, Ingold and co-workers (2,3) had synthesized deuterium-substituted *RRR*- and *SRR*-α-Toc, in which the substituted numbers of deuterium were varied to distinguish them and to elucidate their absorption, transport, and biodiscrimination of *RRR*- and *SRR*-α-Toc. According to their results, the ratio of *RRR*-α-Toc to *SRR*-α-Toc in every tissue (except the liver) was higher than 1.0, suggesting that biodiscrimination occurs in the liver. In these studies, they used a gas chromatography/mass spectrometry-selected ion monitoring method for determining the isomers, because the simpler and less expensive analytical methods had not yet been developed.

In 1993, Ueda *et al.* (4) developed a new chiral high-performance liquid chromatography (HPLC) method to separate eight stereoisomers of α-Toc acetate into four peaks—the first peak was composed of the four 2*R*-isomers, the second peak of *SSS*- and *SSR*-α-Toc, the third peak of *SRR*-α-Toc, and the fourth peak of *SRS*-α-Toc. This HPLC method enabled us to resolve all-*rac*-α-Toc into 2*R*-isomers and three peaks of 2*S*-isomers. *RRR*- and *SRR*-α-Toc are completely separated into two peaks. This method is therefore convenient for elucidating the absorption, transport, and distribution of stereoisomers of α-Toc, especially in animal experiments.

We reported the distribution of α-Toc stereoisomers in the tissues of rats fed diets containing different levels of all-*rac*-α-Toc acetate using this HPLC method. The concentrations of 2*R*-isomers of α-Toc in the blood and tissues of the rats in the all-*rac*-α-Toc acetate groups were significantly higher than the concentration of the 2*S*-isomers. In most tissues, the level of 2*S*-isomers was in order of *SRS* > (*SSS* + *SSR*)/2 > *SRR* (5,6).

Absorption of α-Toc occurs in the presence of bile acids and fatty acids in small intestine. After uptake into intestinal mucosal cells, α-Toc is secreted into chylomicrons in lymph (3,7,8). Chylomicrons are catabolized in the circulation by the lipoprotein lipase, which formed the chylomicron remnants. After uptake of chylomicron remnants by the liver, α-Toc could be metabolized or secreted into very low density lipoprotein (VLDL). Traber *et al.* (9) elucidated the lipoprotein transport and biodiscrimination of two deuterium-labeled stereoisomers (*RRR*- and *SRR*-α-Toc) of α-Toc in healthy human subjects and concluded that no discrimination between the *RRR*- and *SRR*-α-Toc was observed during absorption and chylomicron secretion.

In the present study, we have investigated the biodiscrimination of α-Toc stereoisomers during intestinal absorption. Intestinal absorption of α-Toc stereoisomers was measured in rats with cannulated thoracic ducts, and the uptake of α-Toc stereoisomers was measured using the Caco-2 cells cultured on filter membranes as *in vitro* model.

MATERIALS AND METHODS

Materials. *RRR*-α-Toc acetate, all-*rac* (2*RS*,4′*RS*,8′*RS*)-α-Toc acetate and 2-*ambo*(2*RS*,4′*R*,8′*R*)-α-Tocs (free and acetate) were kindly donated by Eisai Co. Ltd. (Tokyo, Japan).

*To whom correspondence should be addressed at Institute of Environmental Science for Human Life, Ochanomizu University, 2-1-1, Ohtsuka, Bunkyo-ku, Tokyo 112, Japan.

Abbreviations: HPLC, high-performance liquid chromatography; α-Toc, α-tocopherol; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

Animals. Male Sprague-Dawley strain rats (3 weeks of age) were purchased from Nippon Clea Co. (Tokyo, Japan). They were initially fed a commercial diet (CE-2; Nippon Clea Co.) for one week to allow them to adapt to the new environment. These rats were then fed a vitamin E-deficient diet (AIN-76; Eisai Co.) for eight weeks. The vitamin E-deficient diet was composed of 23.68% sucrose, 23.68% glucose, 18.95% vitamin-free casein, 14.21% corn starch, 4.74% filter paper, 3.32% mineral mixture, 0.95% vitamin mixture, 0.28% DL-methionine, 0.19% choline bitartrate, and 10% stripped corn oil. Animals were housed individually in cages at 22°C and 55% humidity, with a 12 h light/dark cycle. The feed and water were supplied *ad libitum.*

Lymphatic cannulation method. Lymphatic cannulation was carried out as described by Ikeda *et al.* (10). Under nembutal anesthesia, vitamin E-deficient rats were subjected to cannulation of the left thoracic lymphatic channel, and an indwelling catheter was placed in the stomach. Normal osmotic solution containing 139 mM glucose and 85 mM NaCl was infused continuously until the end of lymph collection at a rate of 3 mL/h through the gastric tube. After surgery, animals were placed in restraining cages (in the light) and allowed to drink water *ad libitum.* The next morning, after the collection of lymph for 2 h (blank lymph), each animal was administered 3 mL of a test emulsion through the gastric tube and the lymph was collected for a further 24 h. The 3 mL test emulsion contained 200 mg of sodium taurocholate, 50 mg of fatty acid-free albumin, and 200 mg of triolein with 100 mg of *RRR*-α-Toc acetate, all-*rac*-α-Toc acetate, or 2-*ambo*-α-Toc, respectively. The emulsions were prepared by sonication. The lymph was collected in a tube containing EDTA at 3 h intervals during the first 9 h after administration of the test emulsion, and then collected continuously from 9 to 24 h.

Preparation of Caco-2 cells. In recent years there has emerged a cell line that originates from human colon cancer cells, Caco-2. This cell line was established by Fogh *et al.* (11). Caco-2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD), and were grown in plastic flasks at 37°C in air and 5% CO_2 in minimum essential medium containing 10% fetal bovine serum. For subculture the medium was removed, and the cells were detached from the culture flasks with 0.25% trypsin and 0.02% EDTA. Cells were then suspended and seeded onto filters in dish (split ratio 1:2). After confluency cells, 10 mL of the culture medium containing free 2-*ambo*-α-Toc (final concentration 50 μM) dissolved in 10% bovine serum albumin solution was added to the dish (ϕ100 mm). After 6- and 24-h incubation, cells were washed by phosphate-buffered saline, scraped, and sonicated. Protein was measured by the method of Lowry *et al.* (12).

Measurement of α-Toc stereoisomers. The composition of α-Toc stereoisomers in lymph and cells was analyzed to modify the method of Ueda *et al.* (4). Ethanolic pyrogallol solution (6%) was added to each sample. After adding 60% KOH solution to each of the sample tubes, the contents were saponified with occasional shaking at 70°C for 30 min. After cooling in ice water, 1% NaCl solution was added to the saponified medium, and the medium was shaken vigorously with 10% ethyl acetate in hexane for 1 min. The mixture was centrifuged at 3000 rpm for 5 min. The upper layer was collected to extract all stereoisomers of α-Toc. Extracted α-Toc was acetylated by the dry pyridine and acetic anhydride at 50°C for 15 min. After cooling to room temperature, 1% NaCl solution was added and the whole medium was extracted with hexane. Then, hexane layer was collected and evaporated under a stream of nitrogen gas. The residue was dissolved in 98% methanol for the determination by HPLC. Analysis of the acetate of α-Toc stereoisomers was performed by HPLC. The HPLC instrumentation consisted of an LC Module 1 from Waters (Japan Millipore Ltd., Tokyo, Japan) and SK-25 column oven (Nihon Seimitsu Kagaku, Co., Ltd., Tokyo, Japan). The analysis of α-Toc stereoisomers was performed at 38°C using a Chiralpack OP(+) column (250 × 4.6 mm i.d., Daicel Chem. Ind., Tokyo, Japan). The mobile phase was methanol/water (98:2, vol/vol) at a flow rate of 0.48 mL/min. The detection wavelength for α-Toc acetate was 284 nm.

RESULTS

Biodiscrimination between α-Toc stereoisomers during lymphatic transport in rats. The lymphatic flow for 24 h was comparable in the three groups (Table 1). Lymphatic absorption of *RRR*-, all-*rac*-, and 2-*ambo*-α-Toc is shown in Table 2. Absorptions of each of the three forms of vitamin E were approximately 4–9% of that administered for 24 h, and differences between each groups were not significant. Ratios of α-Toc stereoisomers in lymph of all-*rac*-α-Toc group were equivalent, 2*R*-isomers were represented as approximately 50% of absorbed α-Toc (Table 3). Among 2*S*-isomers, amounts of (*SSS*- + *SSR*-)/2, *SRR*- and *SRS*-α-Toc were almost equal.

Moreover, the ratio of *RRR*-/*SRR*-α-Toc in lymph of the 2-*ambo*-α-Toc group was approximately 1.0 (Table 4). Therefore, biodiscrimination of α-Toc stereoisomers did not occur

TABLE 1
Lymph Flow in Rats[a]

Groups	0–3 h (mL)	3–6 h	6–9 h	9–24 h	0–24 h
RRR-α-Toc	4.9 ± 1.2	5.8 ± 0.5	6.0 ± 0.7	27.5 ± 2.2	44.1 ± 3.6
All-*rac*-α-Toc	5.2 ± 1.3	6.3 ± 1.0	6.0 ± 1.1	23.7 ± 3.0	41.1 ± 6.0
2-*ambo*-α-Toc	4.7 ± 1.2	5.3 ± 1.7	5.3 ± 1.4	23.6 ± 4.3	39.0 ± 8.1

[a]Values are means ± SD of 4–6 rats. α-Toc, α-tocopherol.

TABLE 2
Lymph Recovery of *RRR*-, All-*rac*- and 2-*ambo*-α-Toc[a]

Groups	Absorption (%)				
	0–3 h	3–6 h	6–9 h	9–24 h	0–24 h
RRR-α-Toc	0.24 ± 0.03	0.57 ± 0.13	0.67 ± 0.13	2.82 ± 1.39	4.31 ± 1.38
All-*rac*-α-Toc	0.63 ± 0.22	0.78 ± 0.13	0.78 ± 0.37	4.91 ± 2.03	7.10 ± 2.65
2-*ambo*-α-Toc	1.38 ± 0.36	1.45 ± 0.32	1.81 ± 0.65	5.39 ± 3.84	8.95 ± 4.20

[a]Values are means ± SD of 4–6 rats. Abbreviation as in Table 1.

TABLE 3
Ratios of α-Tocopherol Stereoisomers in Lymph of Rats at the All-*rac*-α-Tocopherol Group[a]

Times	2*R*	*SSS* + *SSR*	*SRR*	*SRS*
0–3 h	54%	23%	12%	11%
3–6 h	52%	23%	13%	12%
6–9 h	52%	23%	13%	12%
9–24 h	53%	22%	13%	12%

[a]2*R* consists of *RRR*-, *RRS*-, *RSR*-, and *RSS*-configuration.

TABLE 4
Ratios of α-Tocopherol Stereoisomers in Lymph of Rats at the 2-*ambo*-α-Tocopherol Group

Times	*RRR*-α-Toc	*SRR*-α-Toc
0–3 h	52%	48%
3–6 h	51%	49%
6–9 h	52%	48%
9–24 h	51%	49%

TABLE 5
Incorporation of 2-*ambo*-α-Tocopherol into Caco-2 Cells After 6- and 24 h Incubation[a]

Incubation time	*RRR*-α-Toc	*SRR*-α-Toc
6 h	0.269	0.270
	0.215	0.251
24 h	0.755	0.756
	0.882	0.747

[a]Data expressed as μg/mg protein. Abbreviation as in Table 1.

during absorption in small intestine or during their release into the lymph.

Biodiscrimination of α-Toc stereoisomers in Caco-2 cells. Caco-2 cells were incubated with 2-*ambo*-α-Toc at 37°C for 6 or 24 h. Table 5 shows the concentrations of *RRR*- and *SRR*-α-Toc in Caco-2 cells. The concentration of *RRR*-α-Toc in Caco-2 cells was not significantly different from that of *SRR*-α-Toc. The ratio of *RRR*- and *SRR*-α-Toc in Caco-2 cells at 6 and 24 h was approximately 1:1. Therefore, no discrimination occurred during uptake of 2-*ambo*-α-Toc into Caco-2 cells.

DISCUSSION

We investigated the biodiscrimination of α-Toc stereoisomers during intestinal absorption *in situ* and *in vitro*. By cannulating the thoracic lymph duct, it is possible to obtain a more accurate measurement of the absorbed amount of α-Toc. Traber *et al.* (13) have reported that the absorption of α-Toc was approximately 65% when α-Toc acetate was administered during a slow continuous infusion of soybean oil into the duodenum in rats. In addition, Sugano *et al.* (14) have reported on the intestinal absorption of α-, β-, γ-, and δ-tocotrienol and α-toc in rats with cannulated thoracic ducts. The absorption of these compounds was 10–20% of that administered over 24 h. Absorption of α-Toc-3 was about two times higher than that of α-Toc. According to our results, the absorption of *RRR*-, all-*rac*-, and 2-*ambo*-α-Toc acetate were 4–9% of that administered over 24 h. The lower absorption of α-Toc observed in this study compared to that of Traber *et al.* (13) or Sugano (14) may be due to different methods of administering α-Toc, differences in the length of time the animals had to recover, and differences in the level of dose administered. Among α-Toc stereoisomers, no discrimination was observed during intestinal absorption *in situ*.

The use of cultured Caco-2 cells could allow investigation in detail on the absorptive process of α-Toc. We measured the discrimination of α-Toc stereoisomers using Caco-2 cells cultured on filters. The amounts of *RRR*- and *SRR*-α-Toc were almost equivalent for 6- or 24-h incubation on filters. These results suggested that the discrimination of α-Toc stereoisomers does not occur in small intestine *in vitro*.

We reported that biodiscrimination between α-Toc stereoisomers in rats occurs following intravenous administration (15). According to these results, we presumed that the intravenously administered 2*R*- and 2*S*-isomers were easily transported into the liver from the plasma, the 2*R*-isomers being preferentially released from the liver into the blood, whereas the 2*S*-isomers remained in the liver. Also, Sato *et al.* (16) have reported that two types of purified rat tocopherol-transfer proteins discriminated predominantly against α-Toc from among the α-, β-, γ-, and δ-Toc analogous during transfer between liposomes and mitochondria. Biodiscrimination does not take place in the intestine but at some other site, most likely the liver. Kayden and Traber (17) suggested that this tocopherol-transfer protein could preferentially transport *RRR*-α-Toc to the endoplasmic reticulum for secretion into VLDL during assembly.

In summary, α-Toc stereoisomers are taken up into the intestinal cells without the discrimination, then are secreted equally into chylomicrons and transported to the plasma. In the future, we plan to focus on the tocopherol-transfer pro-

teins in liver as for the biodiscrimination of α-Toc stereoisomers.

ACKNOWLEDGMENTS

We thank Dr. Kunio Ikeda, Kyusyu University School of Agriculture, for leading the lymphatic cannulation method, and Mie Kasuga for her assistance in the preparation of Caco-2 cell line.

REFERENCES

1. Weiser, H., and Vecchi, M. (1982) Stereoisomers of α-Tocopheryl Acetate II. Biopotencies of All Eight Stereoisomers, Individually or in Mixtures, as Determined by Rat Resorption-Gestation Test, *Int. J. Vitam. Nutr. Res. 52*, 351–370.
2. Ingold, K.U., Burton, G.W., Foster, D.O., Hughes, L., Lindsay, D.A., and Webb, A. (1987) Biokinetics of and Discrimination Between Dietary *RRR*- and *SRR*-α-Tocopherols in the Male Rat, *Lipids 22*, 163–172.
3. Traber, M.G., Ingold, K.U., Burton, G.W., and Kayden, H.J. (1988) Absorption and Transport of Deuterium-Substituted *2'R,4'R,8'R*-α-Tocopherol in Human Lipoproteins, *Lipids 23*, 791–797.
4. Ueda, T., Ichikawa, H., and Igarashi, O. (1993) Determination of α-Tocopherol Stereoisomers in Biological Specimens Using Chiral Phase High-Performance Liquid Chromatography, *J. Nutr. Sci. Vitaminol. 39*, 207–219.
5. Nitta, C., Hayashi, K., Ueda, T. and Igarashi, O. (1993) Distribution of α-Tocopherol Stereoisomers in Rats, *Biosci. Biotech. Biochem. 57*, 1406–1407.
6. Nitta-Kiyose, C., Hayashi, K., Ueda, T., and Igarashi, O. (1994) Distribution of α-Toc Stereoisomers in Rats, *Biosci. Biotech. Biochem. 58*, 2000–2003.
7. Peake, I.R., Windmueller, H.G., and Bieri, J.G. (1972) A Comparison of the Intestinal Absorption, Lymph and Plasma Transport, and Tissue Uptake of α- and γ-Tocopherols in the Rat, *Biochim. Biophys. Acta 260*, 679–688.
8. Hatam, L.J., and Kayden, H.J. (1981) The Failure of α-Tocopherol Supplementation to Alter the Distribution Cholesterol in Normal and Hyperlipoproteinemic Persons, *Am. J. Clin. Pathol. 76*, 122–124.
9. Traber, M.G., Burton, G.W., Ingold, K.U., and Kayden, H.J. (1990) *RRR*- and *SRR*-α-Tocopherols Are Secreted Without Discrimination in Human Chylomicrons, But *RRR*-α-Tocopherol Is Preferentially Secreted in Very Low Density Lipoproteins, *J. Lipid Res. 31*, 675–685.
10. Ikeda, I., Imasato, Y., Nagao, H., Sasaki, E., Sugano, M., Imaizumi, K., and Yazawa, K. (1993) Lymphatic Transport of Eicosapentaenoic and Docosahexaenoic Acids as Triglyceride, Ethyl Ester and Free Acid, and Their Effect on Cholesterol Transport in Rats, *Life Sciences 52*, 1371–1379.
11. Fogh, J., Wright, W.C., and Loveless, J.D. (1977) Absence of HeLa Cell Contamination in 169 Cell Lines Derived from Human Tumors, *J. Natl. Cancer Inst. 58*, 209–214.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.I., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
13. Traber, M.G., Kayden, H.J., Green, J.B., and Green, M.H. (1986) Absorption of Water-Miscible Forms of Vitamin E in a Patient with Cholestasis and in Rats, *Am. J. Clin. Nutr. 44*, 914–923.
14. Sugano, M., Ikeda, I., Imaizumi, K., Koba, K., Okamoto, T., Niiya, I., Watanabe, H., Ong, A.S.H., Tan, D.T.S., and Gapor, A. (1992) Nutrition of Tocotrienols and Lipid Metabolism, in *Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications* (Ong, A.S.H., and Packer, L., eds.) pp. 166–177, Basel.
15. Kiyose, C., Muramatsu, R., Ueda, T., and Igarashi, O. (1995) Change in the Distribution of α-Tocopherol Stereoisomers in Rats After Intravenous Administration, *Biosci. Biotech. Biochem. 59*, 791–795.
16. Sato, Y., Hagiwara, K., Arai, H., and Inoue, K. (1991) Purification and Characterization of the α-Tocopherol Transfer Protein from Rat Liver, *FEBS Lett. 288*, 41–45.
17. Kayden, H.J., and Traber, M.G. (1993) Absorption, Lipoprotein Transport and Regulation of Plasma Concentrations of Vitamin E in Humans, *J. Lipid Res. 34*, 343–358.

[Received June 29, 1995; Revision accepted August 14, 1995]

Sesame Seed and Its Lignans Produce Marked Enhancement of Vitamin E Activity in Rats Fed a Low α-Tocopherol Diet

Kanae Yamashita*, Yoshie Iizuka, Tomoko Imai and Mitsuo Namiki

Department of Food and Nutrition, School of Life Studies, Sugiyama Jogakuen University, Nagoya 464, Japan

ABSTRACT: Three series of experiments demonstrated that sesame seed and its lignans cause significant elevation of α-tocopherol content in rats. In Experiment 1, 20% sesame seed (with a negligible amount of α-tocopherol) supplementing 10 (low), 50 (normal), or 250 (high) mg/kg α-tocopherol diets (protein and fat concentrations in diets were adjusted to 200 and 110 g/kg, respectively) all caused a significant increase of α-tocopherol in the blood and tissue of rats. In Experiment 2, groups of rats were fed five different diets: a vitamin E-free control diet, a low α-tocopherol diet, and three low α-tocopherol diets supplemented with 5, 10, and 15% sesame seed. Changes in lipid peroxides in liver, red blood cell hemolysis, and pyruvate kinase activity, as indices of vitamin E deficiency, were examined. These indices were high in the low α-tocopherol diet, whereas supplementation with even 5% sesame seed suppressed these indices completely and caused a significant increase of α-tocopherol content in the plasma and liver. In Experiment 3, two diets containing sesame lignan (sesaminol or sesamin) and low α-tocopherol were tested. Results in both of the sesame lignan-fed groups were comparable to those observed in the sesame seed-fed groups as shown in Experiment 2. These experiments indicate that sesame seed lignans enhance vitamin E activity in rats fed a low α-tocopherol diet and cause a marked increase in α-tocopherol concentration in the blood and tissue of rats fed an α-tocopherol-containing diet with sesame seed or its lignans.

Lipids 30, 1019–1028 (1995).

Sesame seed has long been used as a health food to prevent aging (1,2). However, there have been few detailed analytical studies clarifying such effects. Sesame seed is not only rich in oil (about 50%) and protein (about 20%), but also in characteristic lignans (such as sesamin and sesamolin). Sesame oil is known empirically to be highly stable against oxidative deterioration in cooking. It was found that its highly antioxidative properties appear to be related to its characteristic lignan components (3,4).

We examined the effect of sesame seed with highly antioxidative properties and as yet unclarified characteristics of lignans in senescence by using senescence-accelerated mouse developed by Takeda *et al.* (5) and have found that the advancement of senescence was apparently suppressed by the long-term feeding of sesame seed (6–8). It is to be noted that sesame seed contains only γ-tocopherol but the α-tocopherol content is negligible. γ-Tocopherol has exhibited a stronger antioxidative activity than α-tocopherol in *in vitro* tests, but its vitamin E activity is estimated to be only 6–16% that of α-tocopherol (9), indicating that sesame seed is likely a poor source of vitamin E. In this regard, we have recently found that sesame seed lignans and γ-tocopherol act synergistically to produce almost the same level of vitamin E activity as α-tocopherol in rats (10). This interesting synergism was observed in the treatment without α-tocopherol supplementation. However, previous studies have shown that α- and γ-tocopherol are both absorbed similarly from the intestine but that α-tocopherol is preferentially bound by the transporting protein in the liver, and most γ-tocopherol is excreted through the bile without combining with the transporting protein (11–15). Therefore, it appears to be of value to clarify whether or not sesame seed lignans act synergistically with α-tocopherol to produce higher vitamin E activity in rats fed a low α-tocopherol-containing diet. We found an enhanced vitamin E activity and marked increase of α-tocopherol concentration in the blood and tissue of rats fed a low α-tocopherol-containing diet with sesame seed or its lignans.

MATERIALS AND METHODS

*Materials.*The white sesame seed used (donated by the Shinsei Co., Aichi, Japan) was thoroughly washed, dried, and ground finely in a mixer. The seed from the same batch was used throughout this investigation. α-Tocopherol, γ-tocopherol, and vitamin E homologues used for biochemical analysis, as well as vitamin E-stripped corn oil, were gifts of the Eisai Co. (Tokyo, Japan). A vitamin E-free vitamin mixture (AIN-76A), including choline bitartrate and a mineral mixture, were made according to AIN-76 formulation (16) by Nihon Nosan Kogyo (Yokohama, Japan). Sesaminol (30% ethanol solution; 76.7% purity as solid) and sesamin (crystals, mixture of sesamin and episesamin) were donated by Takemoto Oil & Fat Co. (Aichi, Japan). Solvents used for chromatography were high-performance liquid chromatography (HPLC) grade from Katayama Chemicals Co., Ltd.

*To whom correspondence should be addressed at Department of Food and Nutrition, School of Life Studies, Sugiyama Jogakuen University, 17-3 Hoshigaoka-motomachi, Chikusa-ku, Nagoya 464, Japan.

Abbreviations: ANOVA, analysis of variance; HPLC, high-performance liquid chromatography; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

(Osaka, Japan). Thiobarbituric acid was purchased from Merck (Darmstadt, Germany). Reagents for the measurement of pyruvate kinase activity (EC 2.7.1.40) were all purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). All other chemicals were of analytical grade.

Animals and diets. Three-week-old male Wistar strain rats (Japan SLC Inc., Shizuoka, Japan) were housed individually in stainless-steel wire-mesh cages at 24.5°C and 55% humidity, with a 12-h light/dark cycle. Rats were maintained in accordance with the Guidelines for Animal Experimentation of Nagoya University. Rats were allowed free access to a non-purified diet for 3–5 d and given an experimental diet for 8 wk. The concentration of tocopherol in diets was confirmed by assay immediately after formulation. Diets were stored at −20°C until used.

In Experiment 1, effects of sesame seed on α- and γ-tocopherol concentrations in plasma and tissue were evaluated in rats fed diets containing low (10 mg/kg diet), normal (50 mg/kg diet), or high (250 mg/kg diet) α-tocopherol with or without 20% sesame seed. Thirty rats were equally divided into six groups and fed the diets shown in Table 1 for 8 wk. In the sesame seed-containing diets, the final concentrations of protein, fat, and carbohydate were adjusted to almost the same as in each control diet by reducing the content of casein, stripped corn oil, and corn starch, estimated to be the concentrations of protein (20 g/100 g), fat (50 g/100 g), and residue (30 g/100 g, sugars and dietary fiber, mostly) present in sesame seed (17). Further, fat concentration was adjusted to 110 g/kg diet because 10 g stripped corn oil containing α-tocopherol was added to the 20% sesame seed-containing diets. After overnight food deprivation, the concentration of α- and γ-tocopherol in the plasma, red blood cells, liver, and kidney were determined.

In Experiment 2, we examined the concentrations of α- and γ-tocopherol and the vitamin E status in rats fed low α-tocopherol (10 mg/kg diet) diets supplemented with 0, 5, 10, or 15% sesame seed, and compared the vitamin E status with the control vitamin E-free diet. The composition of diets in Experiments 2 and 3 is shown in Table 2. Thirty rats were divided into five groups and fed the respective diets for 8 wk.

In Experiment 3, we examined the synergistic effect of sesame seed lignans with α-tocopherol by using two kinds of sesame seed lignan: sesaminol and sesamin (each 2 g/kg diet) instead of the sesame seed used in Experiment 2 (Table 2). Sesame seed contains substantial amounts of lignans such as sesamin and sesamolin (Fig. 1), but contains little sesaminol. Commercial sesame oil is of two types, roasted oil and raw salad oil. Raw salad oil is rich in sesaminol, which is formed from sesamolin during the refining process of the extracted oil (3,4). Sesaminol is a strong antioxidant in *in vitro* tests but sesamin is not. Thirty rats were subjected to five groups fed the following diets for 8 wk: (i) vitamin E-free, (ii) low α-tocopherol-containing (10 mg/kg diet), (iii) (low α-tocopherol + sesaminol)-containing, (iv) (low α-tocopherol + sesamin)-containing, or (v) normal α-tocopherol-containing (50 mg/kg diet) diet. In Experiments 2 and 3, we determined the lipid peroxidation in liver, oxidative hemolysis, plasma pyruvate kinase activity, and α- and γ-tocopherol concentrations in plasma and liver as indices of vitamin E status.

After overnight food deprivation, rats were anesthetized with Nembutal (pentobarbital Na), and blood samples were drawn from the heart using heparinized needles and syringes. The liver and kidneys were excised after the liver was perfused with physiological saline. A hemolysis test of red blood cells was conducted immediately after collection of blood samples. Plasma was prepared, and measurements of plasma pyruvate kinase activity were made within 7 h after killing the rats. The red blood cells were washed three times with isotonic 0.008 mol/L phosphate buffered saline. The final hematocrit of red blood cells was made up to about 50%. The remaining plasma, red blood cells, liver, and kidney were kept at −80°C until analysis.

Analysis of samples. Analytical procedures of samples were essentially the same as described by Yamashita *et al.* (10).

TABLE 1
Composition of Experimental Diets

Ingredient	Low α-Toc	Low α-Toc + sesame	Normal α-Toc	Normal α-Toc + sesame	High α-Toc	High α-Toc + sesame
			g/kg Diet			
Casein(-E)[a]	200	160	200	160	200	160
Mineral mixture (AIN-76)	35	35	35	35	35	35
Vitamin mixture [AIN-76(-E)]	10	10	10	10	10	10
Corn starch(-E)[b]	645	585	645	585	645	585
Stripped corn oil with α-Toc	10	10	10	10	10	10
Stripped corn oil	100		100		100	
Sesame seed[c]		200		200		200
α-Toc[d]	0.01	0.01	0.05	0.05	0.25	0.25
γ-Toc[e]	0	0.047	0	0.047	0	0.047

[a]In sesame seed-containing diets, casein content was reduced depending on sesame seed protein concentration which was calculated to be 20 g/100 g.
[b]Composition of 1 kg diet with corn starch. Corn starch was reduced depending on the concentration of ingredients such as carbohydrates in sesame seed.
[c]Roasted white sesame seed.
[d]α-Tocopherol (Toc) was dissolved in 10 g stripped corn oil.
[e]γ-Tocopherol in 200 g sesame seed. Sesame seed contains 23.5 mg/100 g γ-Toc and negligible amounts of α-Toc.

TABLE 2
Composition of Diets (Experiments 2 and 3)

Ingredient	Experiment 2				
	Control (-E)	Low α-Toc	Low α-Toc + 5% sesame	Low α-Toc + 10% sesame	Low α-Toc + 15% sesame
	g/kg diet				
Casein(-E)[a]	200	200	190	180	170
Mineral mixture (AIN-76)	35	35	35	35	35
Vitamin mixture [AIN-76(-E)]	10	10	10	10	10
Corn starch(-E)[b]	655	655	640	625	610
Stripped corn oil[c]	100	100	75	50	25
Crushed sesame seed[d]			50	100	150
α-Toc[e]		0.01	0.01	0.01	0.01
γ-Toc[f]			0.012	0.024	0.036

Ingredient	Experiment 3				
	Control (-E)	Low α-Toc	Low α-Toc + sesaminol	Low α-Toc + sesamin	Normal α-Toc
	g/kg diet				
Casein(-E)[a]	200	200	200	200	200
Mineral mixture (AIN-76)	35	35	35	35	35
Vitamin mixture [AIN-76(-E)]	10	10	10	10	10
Corn starch (-E)[b]	655	655	647	653	655
Stripped corn oil[c]	100	100	100	100	100
α-Toc[d]		0.01	0.01	0.01	0.05
Sesaminol[g]			2		
Sesamin[h]				2	

[a]In sesame seed containing diets, casein content was reduced, depending on the protein concentration which was calculated to be 20 g/100 g.
[b]Composition of 1 kg diets with corn starch. Corn starch was reduced depending on the concentration of ingredients such as carbohydrates in sesame seed and the concentration of sesaminol-dissolving ethanol.
[c]In sesame-seed containing diets, stripped corn oil was reduced depending on the sasame seed lipid concentration, which was calculated to be 50 g/100 g.
[d]Roasted white sesame seed.
[e]α-Tocopherol (Toc) was added dissolving in stripped corn oil.
[f]γ-Toc in sesame seed. Sesame seed contain 23.5 mg/100 g γ-Toc and negligible amounts of α-Toc.
[g]30% Ethanol solution, 76.7% purity as solid.
[h]Crystals, mixture of sesamin and episesamin.

Lipid peroxides in the blood plasma were determined as thiobarbituric acid reacting substances by the fluorometric method of Yagi (18), and those in the liver and kidney by the thiobarbituric acid colorimetric method of Ohkawa *et al.* (19). α-Tocopherol and γ-tocopherol concentrations in the plasma, red blood cell, liver, and kidney were analyzed by HPLC according to the method of Ueda and Igarashi (20). Red blood cell suspension (0.4 mL) and 20% (wt/vol) homogenate of either liver or kidney (1 mL) were pipetted into 10 mL centrifuge tubes with caps and 6% ethanolic pyrogallol (1 mL) and an ethanolic solution of 2,2,5,7,8-pentamethyl-6-chromanol as an internal standard (1 mL) were added. A 60% KOH solution (0.2 mL) was added and saponified at 70°C for 30 min. After adding 1% NaCl solution (4.5 mL), the whole media were extracted with 10% ethylacetate in *n*-hexane (3 mL). After centrifuging, a portion of the hexane layer was evaporated, redissolved in *n*-hexane (200 μL), and injected into the HPLC system. Plasma was treated without saponification.

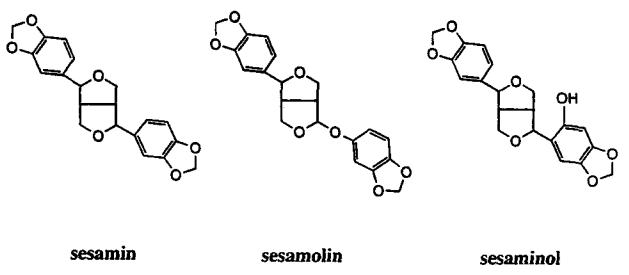


FIG. 1. Sesame seed lignans (sesamin and sesamolin) and related antioxidative compounds (sesaminol). The concentrations of these compounds are as follows: sesamin, 2.5–5.0 mg/g in sesame seed; sesamolin, 1.5–3.0 mg/g in sesame seed; sesaminol 5 μg/g in sesame seed; and 0.5–1.0 mg/g in refined raw sesame oil.

Oxidative hemolysis of red blood cells was measured using dialuric acid (21). Plasma pyruvate kinase activity was determined according to the method described by Gutmann and Bernt (22).

Statistical analysis. Data are expressed as means ± SEM. Experiment 1 was analyzed by two-way analysis of variance (ANOVA) to compare the effect of feeding sesame seed and the effect of dietary α-tocopherol concentration. Subsequently Student's *t*-test was performed to compare the effect

with and without sesame seed. Experiments 2 and 3 were analyzed by one-way ANOVA. When one-way ANOVA revealed $P < 0.05$, the data were further analyzed using Duncan's multiple comparison test (23). Differences were considered statistically significant at $P < 0.05$.

RESULTS

The diets exhibited no significant effect on the animal growth, but we observed significantly greater liver weights in rats fed sesame seed or sesame lignans in Experiments 1, 2, and 3. In Experiment 1, the liver weights in 20% sesame seed-fed groups were significantly higher in comparison with the respective control, 116% in low α-tocopherol, 109% normal α-tocopherol, and 126% high α-tocopherol-fed groups. The relative liver weight in Experiments 2 and 3 is shown in Figure 2. Differences in liver weight corresponded to sesame seed intake (between 5 and 15%). Although both sesamin and sesaminol caused significantly greater liver weight, it was found that sesamin showed a greater effect. The plasma concentrations of α- and γ-tocopherol in Experiment 1 are shown in Figure 3, indicating the level of α-tocopherol was higher in parallel with the dietary concentration of α-tocopherol. Further, sesame seed caused a significant increase in α-tocopherol in the plasma of rats fed diets with low, normal, and high concentrations of α-tocopherol. On the other hand, γ-tocopherol was detected only in the low α-tocopherol + sesame seed-fed groups, but not detected in the normal, as well as high, α-tocopherol-supplemented sesame seed-fed groups. This was invariably observed despite the fact that sesame seed contains about 25 mg/100 g γ-tocopherol and a negligible amount of α-tocopherol. The concentrations of α- and γ-tocopherol in the red blood cells, liver, and kidney in Experiment 1 are shown in Table 3. The concentrations of α-tocopherol in the liver and kidney were low as compared with values reported by other workers (24,25). Sesame seed caused a significantly higher concentration of α-tocopherol in the plasma and tissue of rats fed diets with three α-tocopherol levels (low, normal, or high), as seen in Figure 3. The γ-tocopherol concentrations in the red blood cells, liver, and kidney were detected only in the low α-tocopherol + sesame seed-fed group, and were not detected in the normal and high α-tocopherol with sesame seed fed groups, other than a very small quantity in the liver of rats fed the normal amount of α-tocopherol with sesame seed. In the low α-tocopherol + sesame seed-fed group, the combined concentrations of α-tocopherol and γ-tocopherol in the blood and tissue were almost the same as the concentrations of α-tocopherol in the normal α-tocopherol-fed group. These results may indicate that the supplementation of sesame seed caused an improvement of the vitamin E status of the low α-tocopherol-fed group.

In Experiment 2, we examined whether or not the vitamin E status of the low α-tocopherol-fed group was improved by sesame seed supplementation. We observed that the sesame seed-supplemented groups showed extremely low peroxide concentrations in liver, and even a 5% supplementation with sesame seed to the low α-tocopherol-containing diet almost completely suppressed the high concentration of lipid peroxide in rats fed the low α-tocopherol-containing diet (Fig. 4). Hemolysis and pyruvate kinase activity showed results paralleling peroxide concentrations. The results of Table 4 show that the low α-tocopherol-fed group gave a low concentration

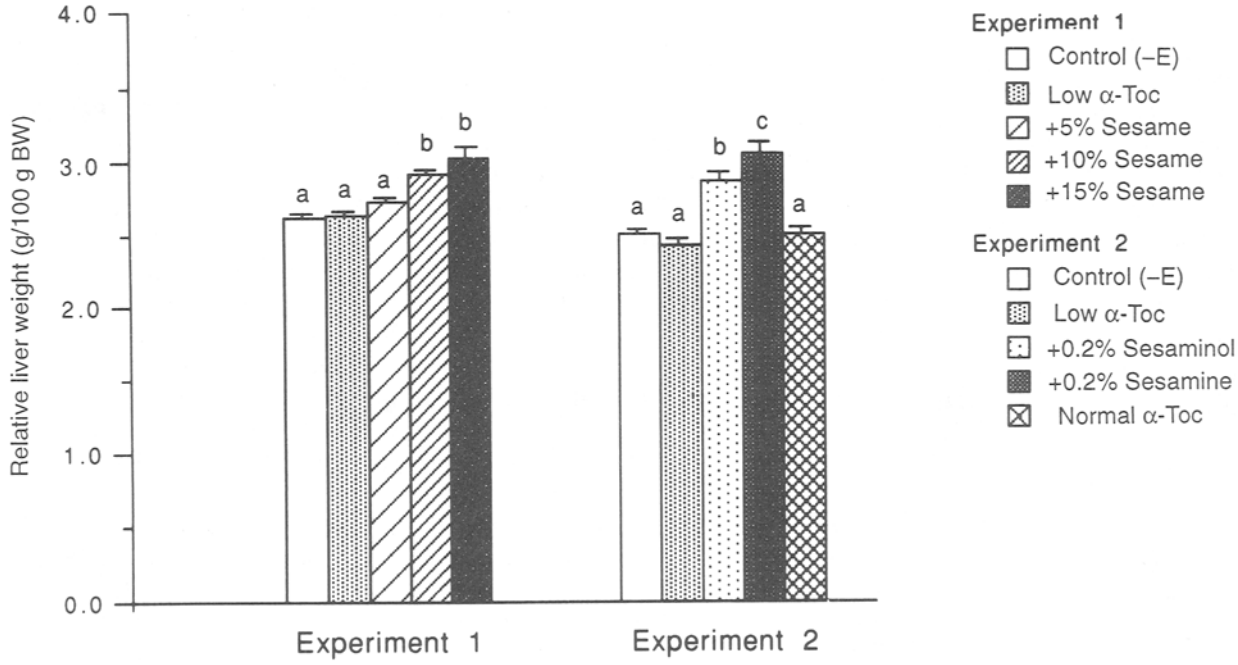


FIG. 2. Effect of sesame seed and its lignans on relative liver weight in rats fed low α-tocopherol (α-Toc) (10 mg/kg diet) diet supplemented with 5, 10, and 15% sesame seed (Experiment 2) or in rats fed low α-tocopherol (10 mg/kg diet) diet supplemented with 2 g sesaminol or sesamin/kg diet (Experiment 3) for 8 wk. Values are means ± SEM, n = 6. Values with different superscripts are significantly different, $P < 0.05$.

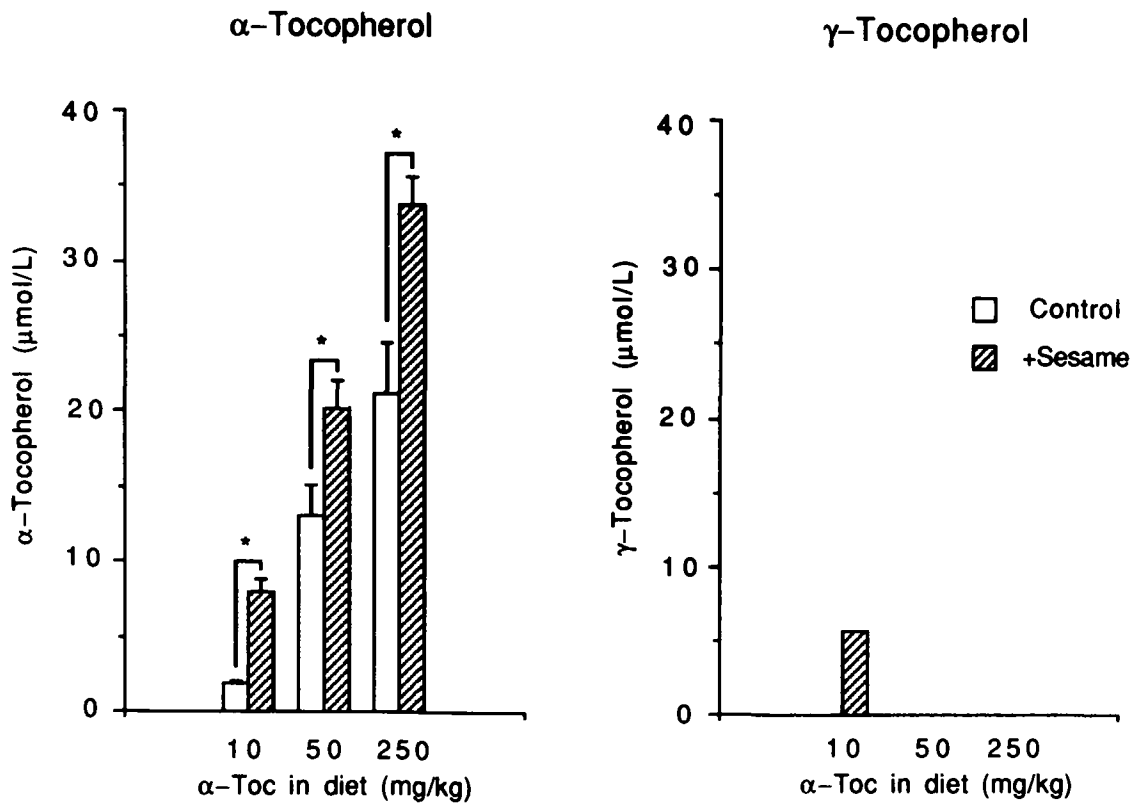


FIG. 3. Plasma α- and γ-tocopherol concentrations in rats fed low (10 mg/kg), normal (50 mg/kg), and high (250 mg/kg) α-tocopherol-containing diets with or without 20% sesame seed. Values are means ± SEM, n = 5. Data were analyzed by two-way analysis of variance. α- and γ-Tocopherol concentrations in plasma were significantly ($P < 0.01$) different among groups fed different concentrations of dietary α-tocopherol, between groups with and without sesame seed, and in their interactions; * shows significantly different, $P < 0.01$ by Student's *t*-test. See Figure 2 for abbreviation.

of α-tocopherol, though it was greater than in the vitamin E-free group; a 5% supplementation of sesame seed (negligible amounts of α-tocopherol) to the low α-tocopherol-containing diet caused significantly higher levels of α-tocopherol and γ-tocopherol. The effect to increase tocopherol level was marked with the 10% supplemented group, and no further increase was observed with the 15% supplemented group except in the α-tocopherol concentration in the liver. The total concentration of α-tocopherol and γ-tocopherol in the plasma and liver in the 10% sesame seed-supplemented group were

TABLE 3
Effect of Sesame Seed on Red Blood Cells (RBC), Liver, and Kidney Concentrations of α- and γ-Tocopherol in Rats Fed a Low, Normal, or High α-Tocopherol-Containing Diets with or without 20% Sesame Seed (Experiment 1)[a]

	Low α-Toc[b]	Low α-Toc + 20% sesame	Normal α-Toc[c]	Normal α-Toc + 20% sesame	High α-Toc[d]	High α-Toc + 20% sesame	*P*-value[e] S	T	S × T
Red blood cell									
α-Tocopherol (μmol/L packed RBC)	1.20 ± 0.09	8.58 ± 0.34[f]	14.00 ± 0.39	18.91 ± 0.60[f]	20.35 ± 1.00	30.23 ± 1.58[f]	0.01	0.01	0.01
γ-Tocopherol (μmol/L packed RBC)	ND	8.47 ± 0.29[f]	ND	ND	ND	ND	0.01	0.01	0.01
Liver									
α-Tocopherol (nmol/g wet liver)	3.55 ± 0.37	22.70 ± 1.67[f]	36.12 ± 1.20	91.59 ± 14.58[f]	92.13 ± 7.63	288.82 ± 54.63[f]	0.01	0.01	0.01
γ-Tocopherol (nmol/g wet liver)	ND	16.53 ± 0.05[f]	ND	1.75 ± 0.40[f]	ND	ND	0.01	0.01	0.01
Kidney									
α-Tocopherol (nmol/g wet kidney)	3.93 ± 0.09	26.70 ± 0.98[f]	30.51 ± 0.63	65.14 ± 0.36[f]	47.56 ± 1.93	91.46 ± 3.07[f]	0.01	0.01	0.01
γ-Tocopherol (nmol/g wet kidney)	ND	24.24 ± 1.13[f]	ND	ND	ND	ND	0.01	0.01	0.01

[a]Values are means ± SEM, n = 5. ND = not detected. [b]Containing 10 mg/kg α-tocopherol (α-Toc). [c]Containing 50 mg/kg α-tocopherol. [d]Containing 250 mg/kg α-tocopherol. [e]S, effect of sesame; T, effect of α-tocopherol concentration; S × T, interaction. [f]$P < 0.01$ for difference between with and without sesame seed in respective α-tocopherol-containing diets.

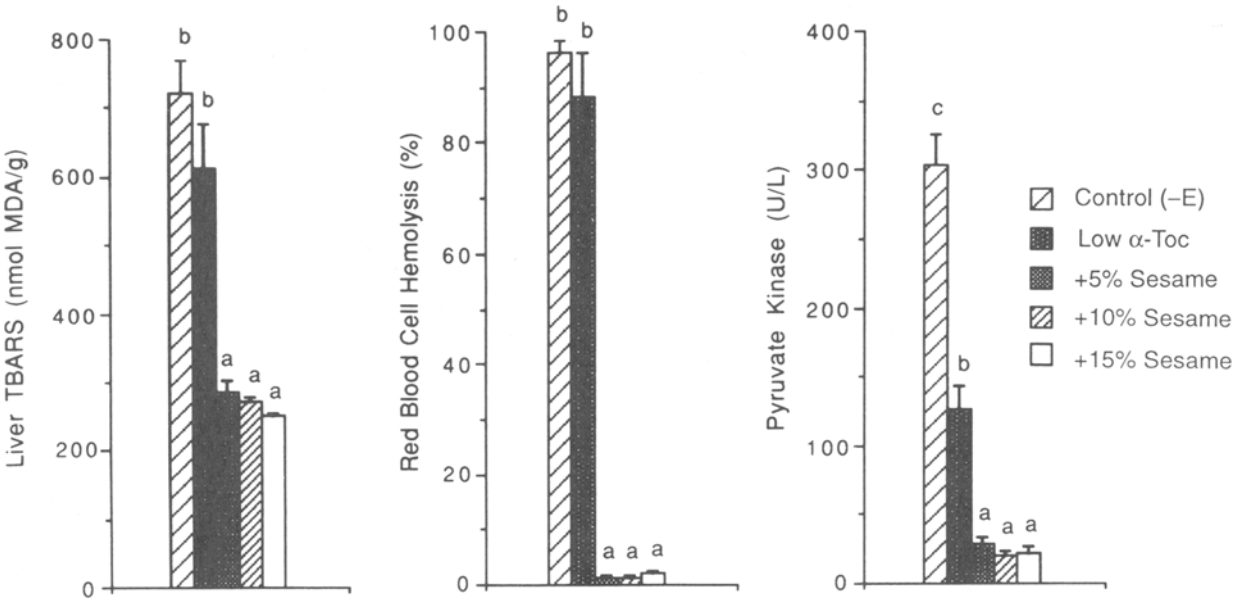


FIG. 4. Effect of sesame seed on thiobarbituric acid reactive substances (TBARS) in liver, red blood cell hemolysis, and plasma pyruvate kinase activity in rats fed low α-tocopherol (10 mg/kg diet) diet supplemented with 5, 10, and 15% sesame seed for 8 wk. Values are means ± SEM, n = 6. Values with different superscripts are significantly different, $P < 0.05$; MDA, malondialdehyde. See Figure 2 for other abbreviation.

found to be nearly the same as that of α-tocopherol in the normal α-tocopherol-fed group in Experiment 1. Although we examined the supplementation effects of sesame seed using a 20% level in Experiment 1, supplementation with 5–10% sesame seed was shown to be sufficient to improve the deficient status of vitamin E in the low α-tocopherol-fed group as shown in Experiment 2.

Experiment 3 was performed using sesaminol and sesamin instead of the sesame seed used in Experiment 2. For the group fed the vitamin E-free diet and the low α-tocopherol-fed group, the results were shown to be essentially the same as in Experiment 2. As shown in Figure 5, hemolysis was nearly absent in the low α-tocopherol + sesaminol-, low α-tocopherol + sesamin-, and normal α-tocopherol-fed groups, in contrast to the high level found in the low α-tocopherol-fed group. Plasma and liver α-tocopherol concentrations in Experiment 3 are shown in Figure 6. They were low in the low α-tocopherol-fed group but slightly higher than in the vitamin E-free group. In the low α-tocopherol + sesaminol- and low α-tocopherol + sesamin-fed groups, significantly greater concentrations of α-tocopherol were found than in the low α-tocopherol diet with neither sesaminol or sesamin, although the concentration was higher in the sesaminol group than in the sesamin group, and lower in both than in the normal α-tocopherol-fed group. Thus it appears evident that both sesaminol and sesamin exhibit the effect of raising α-tocopherol concentrations *in vivo*, and that the antioxidant sesaminol is superior to sesamin (nonantioxidant).

DISCUSSION

We have demonstrated previously that rats fed a vitamin E-free diet supplemented with sesame seed, which contains γ-tocopherol only, exhibited a marked suppression of lipid peroxidation along with an increased γ-tocopherol concentra-

TABLE 4
Effect of Sesame Seed on Plasma and Liver Concentrations of α- and γ-Tocopherol in Rats Fed Low α-Tocopherol Diets Supplemented with 5, 10, and 15% Sesame Seed[a]

	Control (-E)	Low α-Toc[b]	Low α-Toc + 5% sesame	Low α-Toc + 10% sesame	Low α-Toc + 15% sesame
Plasma					
α-Tocopherol (μmol/L)	0.69 ± 0.05[a]	2.14 ± 0.16[b]	5.33 ± 0.30[c]	8.54 ± 0.26[d]	8.37 ± 0.32[d]
γ-Tocopherol (μmol/L)	ND	ND	2.05 ± 0.30[a]	4.50 ± 0.45[b]	4.39 ± 0.76[b]
Liver					
α-Tocopherol (nmol/g wet liver)	1.58 ± 0.07[a]	5.29 ± 0.19[b]	13.91 ± 0.80[c]	20.92 ± 0.91[d]	23.95 ± 0.24[e]
γ-Tocopherol (nmol/g wet liver)	ND	ND	7.40 ± 0.70[a]	14.30 ± 0.98[b]	16.49 ± 1.58[b]

[a]Values are means ± SEM, n = 6. Values with different letters (a–e) in the same line are significantly different, $P < 0.05$. ND = Not detected.
[b]Containing 10 mg/kg α-tocopherol (α-Toc).

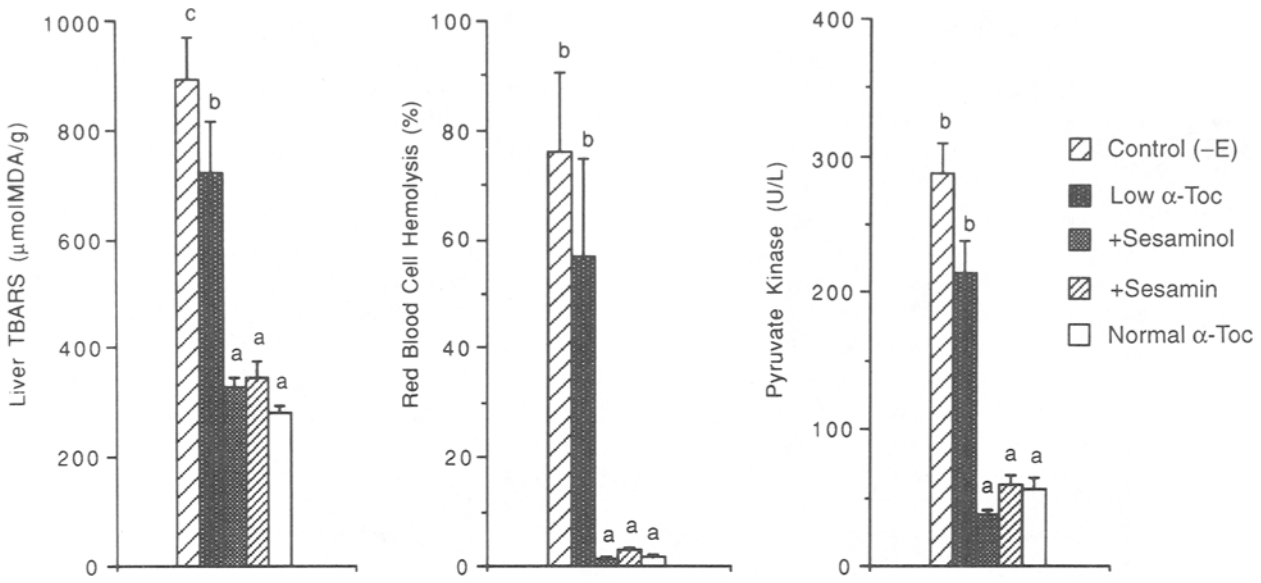


FIG. 5. Synergistic effect of sesame lignans with α-tocopherol *on* lipid peroxides in liver, red blood cell hemolysis, and plasma pyruvate kinase activity in rats fed low α-tocopherol (10 mg/kg diet) diet supplemented with 2 g sesaminol or sesamin/kg diet for 8 wk. Values are means ± SEM, n = 6. Values with different superscripts are significantly different, $P < 0.05$. See Figures 2 and 5 for abbreviations.

tion in the plasma and liver. This indicates that γ-tocopherol in the sesame seed exerts vitamin E activity equal to that of α-tocopherol through a synergistic interaction with sesame seed lignans (10). In the present work, we found that a low α-tocopherol-containing diet (10 mg/kg diet) was able to induce nearly the same vitamin E-deficient status as a γ-tocopherol-containing diet (50 mg/kg diet), and addition of sesame seed or sesame lignans to the low α-tocopherol-containing diet markedly suppressed lipid peroxidation, red blood cell hemolysis, and plasma pyruvate kinase activities, but caused an elevated level of α-tocopherol in blood and tissue (Figs. 4–6; Table 4). We believe that a marked increase of α-tocopherol concentration by sesame seed or sesame lignans *in vivo* is a significant finding since α-tocopherol is thought to be the most important component among its eight homologues in human nutrition (12) and an enhancement of vitamin E activity by other food components has not been reported except in the case of vitamin C (24,26,27), as will be discussed below.

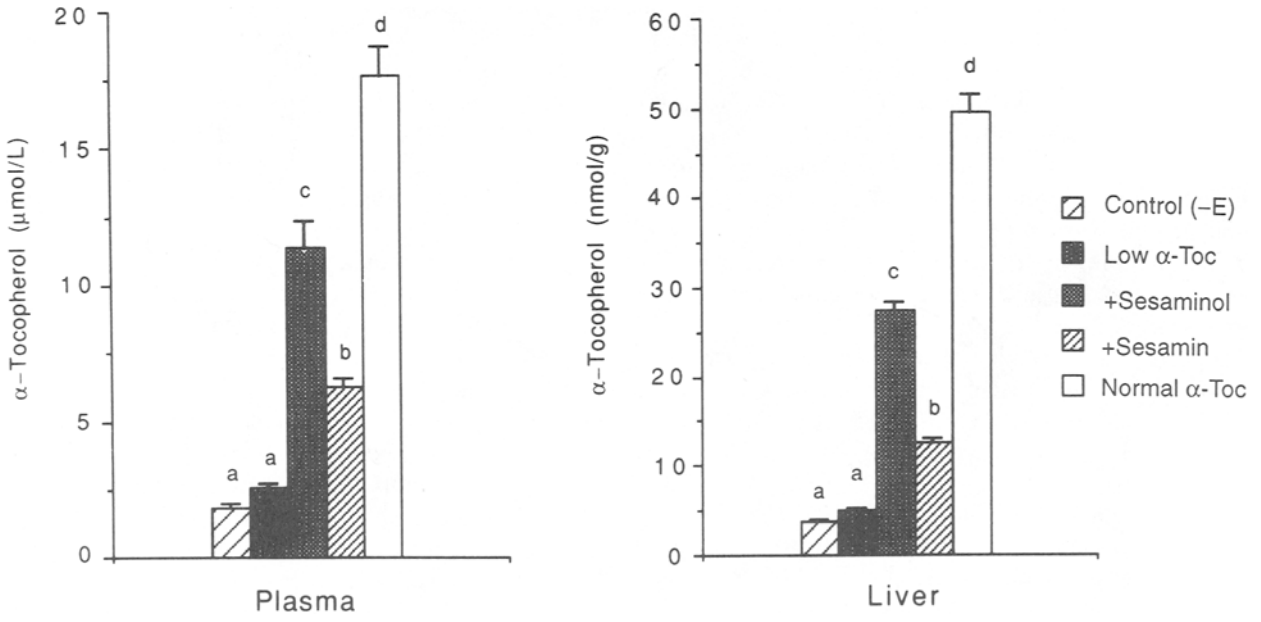


FIG. 6. Synergistic effect of sesame lignans with α-tocopherol on the plasma and liver concentrations of α-tocopherol in rats fed low α-tocopherol diet supplemented with 2 g sesaminol or sesamin/kg diet for 8 wk. Values are means ± SEM, n = 6. Values with different superscripts are significantly different, $P < 0.05$. See Figure 2 for abbreviation.

Concerning the mechanism of this synergistic effect of sesame lignans with α-tocopherol, three possible hypotheses can be put forward: (i) sesame lignan may enhance the binding activity of α-tocopherol with tocopherol-binding protein, (ii) sesame lignan may regenerate α-tocopherol from its oxidized product, and (iii) sesame lignan is a more effective antioxidant than α-tocopherol, protecting the α-tocopherol from oxidation.

Among eight structural forms of vitamin E, it is well known that α-tocopherol is the one found in the highest concentrations in human plasma and tissues, showing the highest biological activities. γ-Tocopherol is most abundant in the common edible vegetable oils, but its biological activity is estimated to be only 6 to 15% that of α-tocopherol. According to recent studies using various forms of vitamin E, it is shown that there is no discrimination between α- and γ-tocopherols during absorption, but following uptake by the liver only RRR-α-tocopherol is preferentially bound to tocopherol-binding protein and secreted in nascent very low density lipoprotein (VLDL) (12). Thus, it is suggested that discrimination between forms of tocopherol may occur on the hepatic tocopherol-binding protein. This was detected only in liver cytosol in rats (15). In our previous experiment, we observed that rats fed γ-tocopherol (50 mg/kg diet) showed very low concentrations of γ-tocopherol in plasma and liver, but it was increased significantly by the addition of sesame lignans to a diet containing the same amount of γ-tocopherol. These results indicate that the binding activity of γ-tocopherol to tocopherol-binding protein is weak, as previously reported by Sato *et al.* (13) and others (14,28,29). However, a possibility cannot be excluded that the presence of sesame lignans may enhance the binding activity of γ-tocopherol to the binding protein.

In Experiment 1, we found higher concentrations of α-tocopherol in the blood and tissues of rats fed diets containing α-tocopherol with sesame seed comparisons in rats fed diets containing only α-tocopherol. Interestingly γ-tocopherol was only detectable in the low α-tocopherol + sesame seed-fed group, but not in the normal or high α-tocopherol + sesame seed groups, despite the fact that the rats had ingested an equal amount of γ-tocopherol. These results may indicate that discrimination between α- and γ-tocopherols exists in rats fed the 20% sesame seed diets supplemented with α-tocopherol. As shown in Table 3, a very low concentration of γ-tocopherol was found only in the liver of rats fed the diet containing the normal α-tocopherol with sesame seed, but not in blood or other tissues. This indicates that γ-tocopherol barely exists in liver following uptake, but it cannot be transported to nascent VLDL due to discrimination of α- and γ-tocopherols by tocopherol-binding protein. These results suggest that the binding activity of tocopherol isomers with tocopherol-binding protein is strongly enhanced by the sesame lignans; but discrimination between α- and γ-tocopherols is given the highest priority.

The second and third possibilities involve antioxidative activity of sesame lignans. In this respect, sesaminol was shown to possess strong antioxidative activity both *in vitro* and *in vivo*. Sesaminol is mainly found in refined raw salad oil but only in small amounts in the sesame seed. Recently Katsuzaki *et al.* (30) reported that there are three new glucosides of sesaminol in defatted sesame cake, and antioxidative activity being generated by treatment with β-glucosidase. It is thus necessary to further investigate whether or not sesamolin, the second major lignan in sesame seed, can possibly be converted to sesaminol *in vivo*.

It is generally agreed that sesamin is not an antioxidant *in vitro*, but recently Asami *et al.* (31) reported that sesamin can be broken down and converted to an antioxidant in liver. Furthermore, we have observed that sesamin is able to almost completely suppress the high level of lipid peroxides in liver caused by a vitamin E-free diet (Yamashita, K., Iizuka, Y., and Namiki, M., unpublished observation). This suggests that sesamin may act as an antioxidant in the liver. The finding that sesaminol, a stronger antioxidant, is superior to sesamin may support these possibilities. There have been several reports indicating the synergistic effects of vitamins E and C (24,26,27), and it has been suggested that antioxidant vitamin C can regenerate vitamin E *in vivo* (24). Recently, we have compared the tocopherol-elevating effect of sesame lignans with that of vitamin C in rats fed a low α-tocopherol diet and found that the former exerted several times stronger activities in comparison to the latter (Yamashita, K., Iizuka, Y., and Namiki, M., unpublished observation: vitamin C increased α-tocopherol level from 1.60 ± 0.14 to 2.31 ± 0.09 μmol/L plasma, from 4.7 ± 0.26 to 7.66 ± 0.41 nmol/g wet liver compared to sesaminol from 2.56 ± 0.14 to 11.40 ± 1.02 μmol/L plasma, from 4.88 ± 0.32 to 27.44 ± 1.02 nmol/g wet liver). However, it is still not yet clear whether a marked increase in α-tocopherol concentration in rats fed diets containing α-tocopherol with sesame lignan can be explained by its possible regeneration from α-tocopherol radical by sesame lignan. Clarification of an antioxidative property of sesaminol is judged to be of great importance in its effect of increasing tocopherol content *in vivo*, because it is a strong antioxidant, and is superior to sesamin in increasing tocopherol levels.

Sesame seed or sesame lignans did not affect the body weight gain, but increased the liver weight. At present, however, it is not yet known whether or not the enlargement of liver weight caused by either sesame seed or its lignans is in favor of animal health, although Sugano *et al.* (32) and Hirose *et al.* (33) also have observed liver enlargement by feeding with sesamin. They reported neither specific histological changes nor accumulation of lipids detectable in the liver, and that the activities of glutamic-oxaloacetic transaminase (GOT EC 2.6.1.1) and glutamic-pyruvic transaminase (GPT EC 2.6.1.2) in serum and drug metabolizing enzymes in the liver all remained unchanged. Recently, several investigations have been reported concerning the physiological function of sesame lignans. For example, Shimizu *et al.* (34) reported that sesamin is a specific inhibitor of Δ5 desaturase, which catalyzes the desaturation of dihomo-γ-linolenic acid (20:3n-6) to yield arachidonic acid. In addition, sesamin exerted a

hypocholesterolemic activity through the inhibition of cholesterol absorption and synthesis (32,33). Furthermore, Akimoto *et al.* (35) reported that sesamin exhibits protective activities in liver injured by continuous inhalation of ethanol or by intraperitoneal administration of carbon tetrachloride. It is still not known whether these observations have any relevance to the enhancing effect on α-tocopherol levels we herewith report in this investigation.

In the present experiment, we have found that sesame seed and sesame lignans cause a high α-tocopherol concentration in plasma and tissues in rats. Based on the results of this investigation, we propose that one possible rationale for the consideration of sesame seed as a health food may exist in its enhancing effect on vitamin E activity. However, more thorough experiments are needed to clarify the nature of interactions between sesame lignans and tocopherol-binding protein.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid for scientific research (C) from the Ministry of Education, Science and Culture (No. 05680040) and by grants of T. Nanba Memorial Health Care Foundation and the Iijima Memorial Foundation for Promotion of Food Science and Technology. We are grateful to Yuuko Kondo and Yumi Maekawa for their technical assistance.

REFERENCES

1. Budowski, P., and Markley, K.S. (1951) The Chemical and Physiological Properties of Sesame Oil, *Chem. Rev. 48*, 125–151.
2. Namiki, M., and Kobayashi, T. (1989) *Science of Sesame*, Asakura Shoten, Tokyo, Japan.
3. Fukuda, Y., Nagata, M., Osawa, T., and Namiki, M. (1986) Contribution of Lignan Analogues to Antioxidative Activity of Refined Unroasted Sesame Seed Oil, *J. Am. Oil Chem. Soc. 63*, 1027–1031.
4. Fukuda, Y., Osawa, T., Namiki, M., and Ozaki, T. (1985) Studies on Antioxidative Substances in Sesame Seed, *Agric. Biol. Chem. 49*, 301–306.
5. Takeda, M.G., Hosokawa, M., Takeshita, S., Irino, M., Higuchi, K., Matsushita, T., Tomita, Y., Yasuhira, K., Hamamoto, H., Shimizu, K., Ishii, M., and Yamamuro, T. (1981) A New Murine Model of Accelerated Senescence, *Mech. Ageing Dev. 17*, 183–194.
6. Namiki, M., Yamashita, K., and Osawa, T. (1993) Food-Related Antioxidants and Their Activities *in vivo*, in *Active Oxygens, Lipid Peroxides and Antioxidants* (Yagi, K., ed.), pp. 319–332, Japan Scientific Society Press, Tokyo/CRC Press, Boca Raton.
7. Yamashita, K., Kawagoe, Y., Nohara, Y., Namiki, M., Osawa, T., and Kawagishi, S. (1990) Effects of Sesame in the Senescence-Accelerated Mouse, *J. Jpn. Soc. Nutr. Food Sci. 43*, 445–449.
8. Yamashita, K., and Namiki, M. (1994) Suppressing Effect of Sesame Seed and Its Lignans on Senescence in Senescence-Accelerated Mouse (SAMP1), in *The SAM Model of Senescence* (Takeda, T., ed.), pp. 153–156, Excerpta Medica-Amsterdam-London-NewYork-Tokyo.
9. Peake, I.R., and Bieri, J.G. (1971) Alpha- and Gamma-Tocopherol in the Rat: *in vitro* and *in vivo* Tissue Uptake and Metabolism, *J. Nutr. 101*, 1615–1622.
10. Yamashita, K., Nohara, Y., Katayama, K., and Namiki, M. (1992) Sesame Seed Lignans and γ-Tocopherol Act Synergistically to Produce Vitamin E Activity in Rats, *J. Nutr. 122*, 2440–2446.
11. Catignani, G.L., and Bieri, J.G. (1977) Rat Liver α-Tocopherol Binding Protein, *Biochim. Biophys. Acta 497*, 349–357.
12. Kayden, H.J., and Traber, M.G. (1993) Absorption, Lipoprotein Transport, and Regulation of Plasma Concentrations of Vitamin E in Humans, *J. Lipid Res. 34*, 343–358.
13. Sato, Y., Hagiwara, K., Arai, H., and Inoue, K. (1991) Purification and Characterization of the α-Tocopherol Transfer Protein from Rat Liver, *FEBS Lett. 288*, 41–45.
14. Traber, M.G., and Kayden H.J. (1989) Preferential Incorporation of α-Tocopherol vs. γ-Tocopherol in Human Lipoproteins, *Am. J. Clin. Nutr. 49*, 517–526.
15. Yoshida, H., Yusin, M., Kuhlenkamp, J., Hirano, A., Stolz, A., and Kaplowitz, N. (1992) Identification, Purification, and Immunochemical Characterization of a Tocopherol-Binding Protein in Rat Liver Cytosol, *J. Lipid Res. 33*, 343–350.
16. American Institute of Nutrition (1977) Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies, *J. Nutr. 107*, 1340–1348.
17. Namiki, M. (1995) The Chemistry and Physiological Functions of Sesame, in *Food Reviews International 11*, 281–329.
18. Yagi, K. (1976) A Simple Fluorometric Assay for Lipoperoxide in Blood Plasma, *Biochem. Med. 15*, 212–216.
19. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction, *Anal. Biochem. 95*, 351–358.
20. Ueda, T., and Igarashi, O. (1987) New Solvent System for Extraction of Tocopherols from Biological Specimens for HPLC Determination and the Evaluation of 2,2,5,7,8,-Pentamethyl-6-Chromanol as an Internal Standard, *J. Micronutr. Anal. 3*, 185–198.
21. Mino, M., Kitagawa, M., and Nakagawa, S. (1981) Changes of α-Tocopherol Levels in Red Blood Cells and Plasma with Respect to Hemolysis Induced by Dialuric Acid in Vitamin E-Deficient Rats, *J. Nutr. Sci. Vitaminol. 27*, 199–207.
22. Gutmann, I., and Bernt, E. (1974) Pyruvate Kinase Assay in Serum and Erythrocytes, in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.), Vol. 2, pp. 774–778. Academic Press, New York.
23. Duncan, D.B. (1957) Multiple Range Tests for Correlated and Heteroscedastic Means, *Biometrics 13*, 164–176.
24. Igarashi, O., Yonekawa, Y., and Fujiyama-Fujihara, Y. (1991) Synergistic Action of Vitamin E and Vitamin C *in vivo* Using a New Mutant of Wistar-Strain Rats, ODS, Unable to Synthesize Vitamin C, *J. Nutr. Sci. Vitaminol. 37*, 359–369.
25. Javouhey-Donzel, A., Guenot, L., Maupoil, V., Rochette, L., and Rocquelin, G. (1993) Rat Vitamin E Status and Heart Lipid Peroxidation: Effect of Dietary α-Linolenic Acid and Marine n-3 Fatty Acids, *Lipids 28*, 651–655.
26. Ingold, K.U., Bowry, V.W., Stocker, R., and Walling, C. (1993) Autoxidation of Lipids and Antioxidation by α-Tocopherol and Ubiquinol in Homogeneous Solution and in Aqueous Dispersions of Lipids: Unrecognized Consequences of Lipid Particle Size as Exemplified by Oxidation of Human Low Density Lipoprotein, *Proc. Natl. Acad. Sci. USA 90*, 45–49.
27. Packer, J.E., Slater, T.F., and Willson, R.L. (1979) Direct Observation of a Free Radical Interaction Between Vitamin E and Vitamin C, *Nature (Lond.) 278*, 737–738.
28. Behrens, W.A., and Madere, R. (1983) Interrelationship and Competition of α- and γ-Tocopherol at the Level of Intestinal Absorption, Plasma Transport and Liver Uptake, *Nutr. Res. 3*, 891–897.
29. Behrens, W.A., and Madere, R. (1987) Mechanisms of Absorption, Transport and Tissue Uptake of RRR-α-Tocopherol and d-γ-Tocopherol in the White Rat, *J. Nutr. 117*, 1562–1569.

30. Katsuzaki, H., Kawagishi, S., and Osawa, T. (1994) Sesaminol Glucosides in Sesame Seeds, *Phytochemistry 35,* 773–776.
31. Asami, S., Akimoto, K., Abe, K., Akamatsu, T., Konishi, K., Shimizu, S., Sugano, M., and Yamada, H. (1993) Antioxidant Activity of Sesamin on NADPH-Dependent Lipid Peroxidation in Liver Microsomes, *Nippon Nogeikagaku Kaishi 67,* 265 (abs.).
32. Sugano, M., Inoue, T., Koba, K., Yoshida, K., Hirose, N., Shinmen, Y., Akimoto, K., and Amachi, T. (1990) Influence of Sesame Lignans on Various Lipid Parameters in Rats, *Agric. Biol. Chem. 54,* 2669–2673.
33. Hirose, N., Inoue, T., Nishihara, K., Sugano, M., Akimoto, K., Shimizu, S., and Yamada, H. (1991) Inhibition of Cholesterol Absorption and Synthesis in Rats by Sesamin, *J. Lipid Res. 32,* 629–638.
34. Shimizu, S., Akimoto, K., Shinmen, Y., Kawashima, H., Sugano, M., and Yamada, H. (1991) Sesamin is a Potent and Specific Inhibitor of Δ5 Desaturase in Polyunsaturated Fatty Acid Biosynthesis, *Lipids 26,* 512–516.
35. Akimoto, K., Kitagawa, Y., Akamatsu, T., Hirose, N., Sugano, M., Shimizu, S., and Yamada, H. (1993) Protective Effects of Sesamin Against Liver Damage Caused by Alcohol or Carbon Tetrachloride in Rodents, *Ann. Nutr. Metab. 37,* 218–224.

[Received April 17, 1995, and in final revised form July 31, 1995; Revision accepted August 14, 1995]

Suppression by Carotenoids of Microcystin-Induced Morphological Changes in Mouse Hepatocytes

Rie Matsushima-Nishiwaki[a], Yoshihiro Shidoji[a], Shinji Nishiwaki[a], Tetsuya Yamada[b], Hisataka Moriwaki[a] and Yasutoshi Muto[a,*]

[a]First Department of Internal Medicine and [b]Second Department of Pathology, Gifu University School of Medicine, Gifu 500, Japan

ABSTRACT: Microcystin-LR is a liver tumor promoter in the okadaic acid class, a group of potent inhibitors of protein phosphatases 1 and 2A. Because of inhibition of protein phosphatases, microcystin-LR induces hyperphosphorylation of cellular proteins, including cytoskeletal proteins—cytokeratins 8 and 18—and causes morphological changes in mouse hepatocytes in primary culture. We studied the effects of carotenoids to antagonize microcystin-LR-induced morphological changes in hepatocytes. β-Carotene (100 nM to 100 μM) suppressed the morphological changes induced by 100 nM microcystin-LR in a dose-dependent manner. Other carotenoids tested exerted similar suppressive effects, although retinoids, such as all-*trans* retinol, all-*trans* retinoic acid, and 9-*cis* retinoic acid, were only weakly suppressive. The relative potency of the suppression correlated significantly with the number of conjugated double bonds in the *trans* configuration. β-Carotene strongly suppressed the hyperphosphorylation of cellular proteins induced by microcystin-LR without significant changes in the basal phosphorylation level. Other antioxidants, such as α-tocopherol, did not protect the cells against microcystin-LR. Taken together, the antagonistic effects of carotenoids against microcystin-LR are difficult to explain by their antioxidant or provitamin A activities. Suppression of the hyperphosphorylation of cellular proteins may be a novel mechanism by which carotenoids inhibit tumor promotion.
Lipids 30, 1029–1034 (1995).

Anticarcinogenic effects of carotenoids have been reported in studies using animal and cellular models and in several human intervention trials of carotenoids for cancer prevention (1–13). The anticarcinogenic actions of carotenoids are, at present, explained mainly by their antioxidative activities (as quenchers of singlet oxygen and free radical scavengers) or by their activities as provitamin A (1,2). However, some nonprovitamin A carotenoids also protect against carcinogenesis, and the radical scavenger activity of carotenoids does not always correlate with their anticarcinogenic effect (4,5,14). Therefore, biological mechanisms other than provitamin A activities or antioxidative effects may be operative in the suppression of cancer development by carotenoids.

In the process of multiple-step carcinogenesis, the promotion phase is the longest and can be retarded or even reversed by repeated applications of anti-tumor agents. Recently, okadaic acid class tumor promoters were reported to promote tumor formation in various organs by potently inhibiting protein phosphatases 1 and 2A (15–17). Microcystin-LR, a hepatotoxic compound produced by a planktonic cyanobacteria, is a liver tumor promoter of the okadaic acid class (17). Microcystin-LR strongly inhibits the activities of protein phosphatases 1 and 2A in hepatocytes (18), and subsequently induces various responses, such as protein hyperphosphorylation, morphological changes of hepatocytes (18), and tumor promotion (17). Schwartz *et al.* (19–21) reported that β-carotene induced expression of the cdc2 kinase (one of a cell cycle regulator), cyclins A and D, wild-type p53 and stress proteins; and also repressed expression of mutant p53, c-*Fos*, and *Raf*-1. They also found reduction of glutathione-S-transferase activity, superoxide dismutase activity, and the level of nonprotein sulfhydryls in the tumor cells by β-carotene (19,22). These modifications of intracellular signals by β-carotene may correlate with significant alterations in signal transductions, such as protein phosphorylation, in the tumor cells.

In the present study, we investigated whether carotenoids suppress the activities of okadaic acid class tumor promoters and found that carotenoids antagonized the morphological changes in mouse hepatocytes in primary culture induced by microcystin-LR. We also found that β-carotene suppressed the hyperphosphorylation of proteins induced by microcystin-LR in the hepatocytes.

EXPERIMENTAL PROCEDURES

Chemicals. A *dunariella* preparation containing (% by weight) 2% β-carotene, 0.3% 9-*cis* β-carotene (purity 86%, mainly contaminated with all-*trans* β-carotene), 0.2% astaxanthin, and 0.2% sulfated astaxanthin in liposomes was obtained from Kyowa Hakko Ltd. (Tokyo, Japan). The liposomes contained 61% glycerol, 5% esterified glycerol, 1%

*To whom correspondence should be addressed at First Department of Internal Medicine, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan.

Abbreviations: DMSO, dimethylsulfoxide; THF, tetrahydrofuran.

Copyright © 1995 by AOCS Press

lecithin, and 48 μM α-tocopherol. The *dunariella* β-carotene consisted of 50% all-*trans* and 50% 9-*cis* forms. The mean diameter of the mixed micelles in the emulsion was approximately 0.1 μm. All-*trans* β-carotene, lutein, lycopene, retinol, and all-*trans* retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 9-*Cis* retinoic acid was obtained from Kuraray Co. Ltd. (Okayama, Japan). Fucoxanthin and canthaxanthin were obtained from Roche Japan Ltd. (Tokyo, Japan). Microcystin-LR was a generous gift from Dr. Hirota Fujiki, Saitama Cancer Center Research Institute (Saitama, Japan). Okadaic acid was purchased from Wako Pure Chemical Co. (Osaka, Japan). ^{32}P-Orthophosphate was obtained from Amersham Co. (Buckinghamshire, United Kingdom).

Preparation of mouse primary culture hepatocytes. Eight-week-old male Balb/c mice were obtained from Chubu Kagaku Shizai Co. (Nagoya, Japan). Hepatocytes were isolated from mouse liver digested with collagenase; 1×10^5 cells/500 μL were inoculated on collagen-precoated 24-well plastic dishes to evaluate morphological changes, and 1×10^6 cells/mL were inoculated on collagen pretreated 6-well plastic dishes to analyze the phosphorylation of cellular proteins (18). The hepatocytes were cultured in William's E medium containing 5% fetal calf serum, 1 nM insulin, and 1 nM dexamethasone at 37°C in an atmosphere containing 5% CO_2.

Morphological observation of primary culture hepatocytes. After overnight culture of mouse hepatocytes, 100 nM microcystin-LR or 70 nM okadaic acid was added to the media and followed immediately by adding a test carotenoid or retinoid. The concentration of test compounds was as high as 50 μM for emulsified β-carotene, astaxanthin, or sulfated astaxanthin, and as high as 10 μM for other carotenoids or retinoids. Nonemulsified compounds were dissolved in a 1:1 solution of dimethylsulfoxide (DMSO) and tetrahydrofuran (THF). DMSO alone was added as a negative tumor promoter control, and DMSO/THF (1:1, vol/vol) or vehicle emulsion was added for each test. The final fractional volumes of the emulsion and DMSO + THF in the media were below 1 and 0.8%, respectively. After 3 h of incubation, a microphotograph of cells in each well was taken. Approximately 200 hepatocytes were counted on the microphotograph. The rate of suppression of morphological changes (%) was calculated by using the control being treated only with vehicle (100%) and treated only with tumor promoter (0%).

Measurement of phosphorylation of cellular proteins in primary culture hepatocytes. Mouse hepatocytes were preincubated in primary culture overnight in a phosphate-free Dulbecco's modified Eagle's medium with 1.85 MBq/mL ^{32}P-orthophosphate in each well. Then, 100 nM microcystin-LR was added, and incubation was continued for another 3 h in the presence or absence of β-carotene. The cells were scraped and solubilized in 200 μL of lysis buffer consisting of 50 mM 4-(2-hydroxyethyl)-l-piperazinediethanesulfonic acid (pH 7.4), 250 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethanesulfonyl fluoride (23). The lysate (20 μL) was subjected to 10% SDS–polyacrylamide gel electrophoresis according to the method of Laemmli (24), and phosphorylated proteins were detected by autoradiography. Densitometric measurement of each autoradiograph was performed using an image analyzer (GT6000; Epson, Tokyo, Japan).

Indirect immunofluorescence analysis of cytokeratin 18. Primary culture mouse hepatocytes (5×10^4 cells/0.5 mL/chamber) were plated in a 4-chamber glass slide (Nunc Inc., Naperville, IL) and treated with 100 nM microcystin-LR with or without 10 μM β-carotene for 2 h. After fixation with cooled acetone at −20°C, the cells were incubated with anti-cytokeratin 18 antibody (ImnoMAb™CK#18.04; IBL Research Products Co., Cambridge, MA) for 1 h at 30°C. After incubating for 30 min with an FTTC-conjugated second antibody, the slides were examined with a fluorescence microscope (Axioskop MC 100; Karl Zeiss, Okerkochen, Germany) (25).

RESULTS

Suppression of morphological changes of primary culture mouse hepatocytes. Within 3 h of incubation, morphological changes were observed in approximately 60% of the hepato-

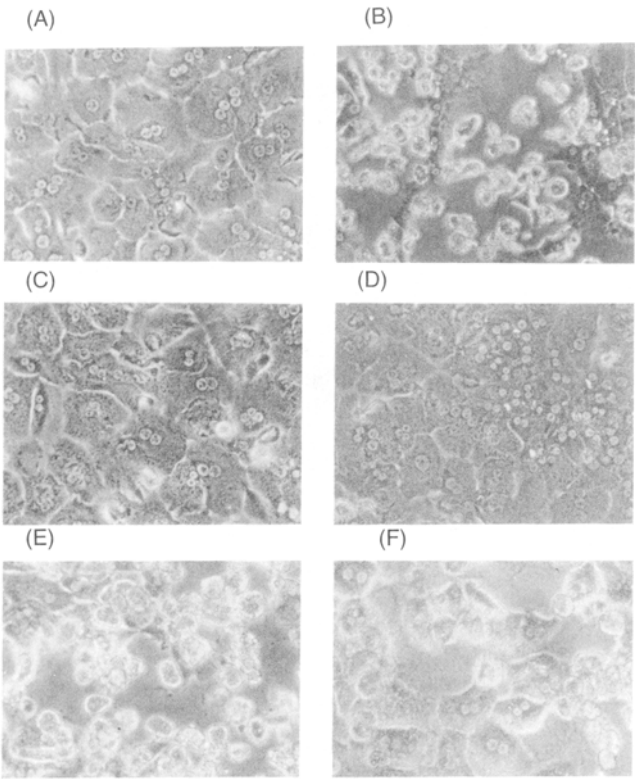


FIG. 1. β-Carotene or lutein protected the mouse primary culture hepatocytes from microcystin-LR-induced morphological changes (×152). After 3 h of incubation, morphological changes were observed in the hepatocytes treated with 100 nM microcystin-LR (B) or 70 nM okadaic acid (E). However, 10 μM β-carotene (C) and 10 μM lutein (D) with microcystin-LR or 10 μM β-carotene with okadaic acid (F) showed reduction of the morphological changes of the cells. (A) control (vehicle alone); (B) 100 nM microcystin-LR; (C) 10 μM β-carotene and 100 nM microcystin-LR; (D) 10 μM lutein and 100 nM microcystin-LR; (E) 70 nM okadaic acid; and (F) 10 μM β-carotene and 70 nM okadaic acid.

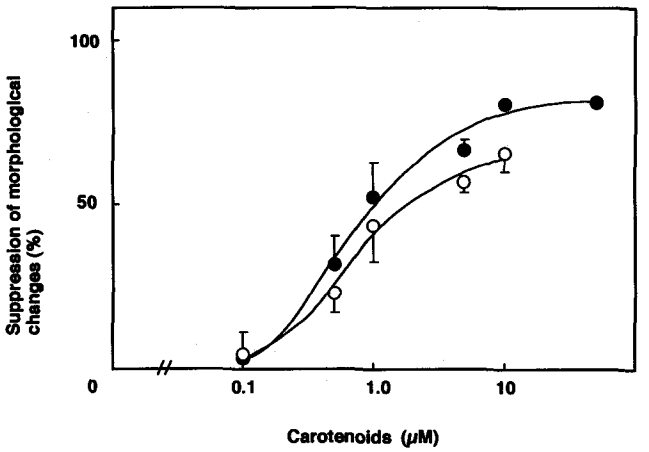


FIG. 2. Concentration dependence of the suppressive effect of carotenoids on microcystin-LR-induced morphological changes of mouse primary culture hepatocytes. The rates of suppression of morphological changes of hepatocytes induced by 100 nM microcystin-LR and the control were taken as 0 and 100%, respectively. Data represent the means ± SD of four determinations; ●, β-carotene, ○, lutein.

cytes treated with 100 nM microcystin-LR (Fig. 1B). Ten μM β-carotene or lutein reduced the effect of microcystin-LR on the morphology of the hepatocytes (Fig. 1C, 1D). β-Carotene also suppressed okadaic acid-induced morphological changes in the hepatocytes, although the effect was weaker than that on microcystin-LR-induced morphological changes (Fig. 1E, 1F). The vehicles of β-carotene or lutein alone did not have any effect on morphological changes of microcystin-LR-treated or nontreated hepatocytes (Fig. 1A).

β-Carotene and lutein dose-dependently suppressed microcystin-LR-induced morphological changes at concentrations from 100 nM to 10 μM (Fig. 2). When the extent of suppression of the morphologically changed hepatocytes, which were treated with 100 nM microcystin-LR alone and with solvent alone, were 0 and 100%, respectively, the 50% inhibitory doses (ID_{50}) of β-carotene and lutein were 0.9 and 2.1 μM, respectively (Fig. 2).

Other carotenoids also were observed to protect against morphological changes of the hepatocytes. With the suppressive activity of 10 μM *dunariella* β-carotene considered as 1.0, the relative potencies of suppression by other carotenoids and retinoids were calculated using the following equation: relative potency of suppression = [extent (%) of suppression of morphological changes with 10 μM carotenoid or retinoid]/[extent (%) of suppression of morphological changes with 10 μM *dunariella* β-carotene]. A significant correlation was observed between the number of the *trans*-conjugated double bonds of the compounds and the relative suppression of the morphological changes (Fig. 3). For example, the relative potencies of all-*trans* β-carotene, canthaxanthin, astaxanthin, and sulfated astaxanthin, which have 13 double bonds, were 1.07–1.17, and those of 9-*cis* β-carotene, which have nine conjugated double bonds in *trans* form, were 0.73, and lutein and α-carotene, which have ten conjugated double bonds, were 0.64–0.81.

Phosphorylation of cellular proteins in primary culture hepatocytes. Treatment with microcystin-LR for 3 h increased the phosphorylation of several proteins in the hepatocytes (Fig. 4, lane 2), and β-carotene particularly reduced the hyperphosphorylation of 55 and 49 kDa proteins (Fig. 4, lanes 3–5). However, hepatocytes treated with as much as 100 μM β-carotene alone did not show detectable changes in the level of protein phosphorylation as compared to the control (Fig. 4, lane 6). Densitometry of the 55 and 49 kDa bands revealed that β-carotene dose-dependently suppressed the hyperphos-

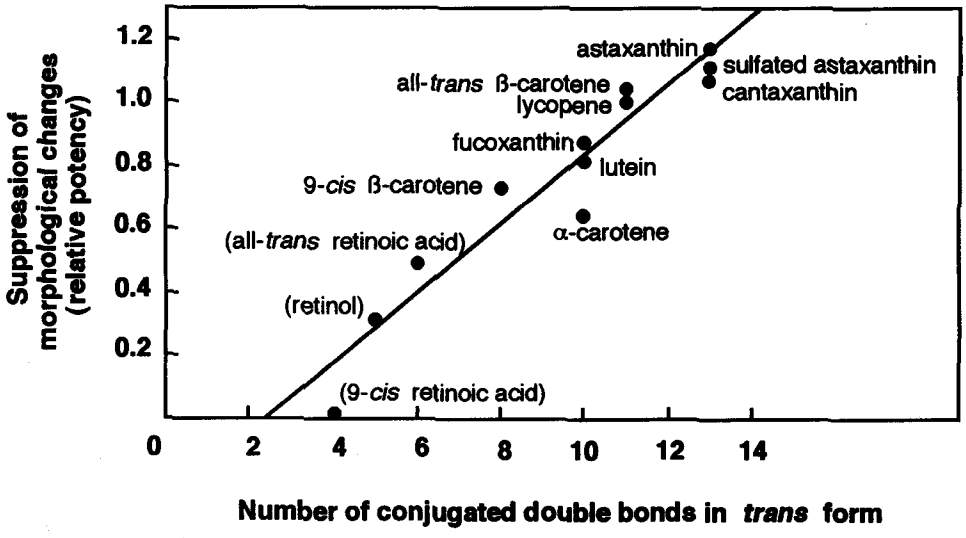


FIG. 3. Relative potency of suppression of 100 nM microcystin-LR-induced morphological changes of primary culture mouse hepatocytes by 10 μM carotenoids. See text for the definition of relative potency of suppression of morphological changes. The X axis represents the number of the *trans*-conjugated double bonds in each compound. Data represent the means of three determinations: $Y = 0.1086 \times -0.2583$, $r = 0.9589$, $P < 0.001$.

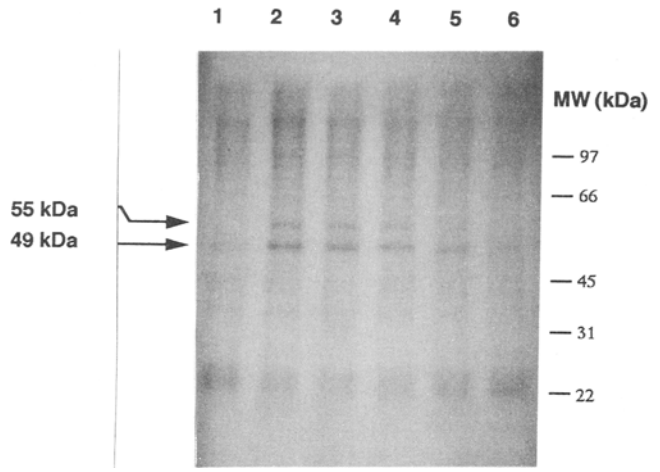


FIG. 4. Suppression by β-carotene of microcystin-LR-induced hyperphosphorylation of cellular proteins in primary cultured mouse hepatocytes. ^{32}P-labelled cellular proteins were separated by SDS–polyacrylamide gel electrophoresis and detected by autoradiography. Lane 1, control; lane 2, 100 nM microcystin-LR; lanes 3, 4, and 5, 1 μM, 10 μM, and 100 μM β-carotene, respectively, with 100 nM microcystin-LR; lane 6, 100 μM β-carotene alone.

phorylation of these proteins induced by 100 nM microcystin-LR (Fig. 5).

Indirect immunofluorescence microscopy. Treatment of the hepatocytes with 100 nM microcystin-LR caused significant retraction of cytokeratin 18 filaments (Fig. 6B). Ten μM

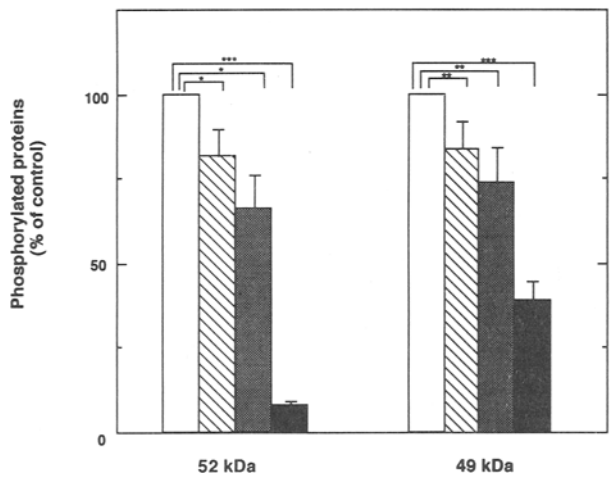


FIG. 5. Densitometric demonstration of the suppressive effect of β-carotene on hyperphosphorylation of 49 and 55 kDa proteins induced by microcystin-LR in primary cultured mouse hepatocytes. Density of the 100 nM microcystin-LR phosphorylated 49 and 54 kDa protein bands is 100% (white bar). One μM (striped bar), 10 μM (gray bar), 100 μM (black bar) β-carotene cotreated with 100 nM microcystin-LR showed reduction of the hyperphosphorylations of 49 and 55 kDa proteins dose-dependently. White bar, 100 nM microcystin-LR; striped bar, 1 μM β-carotene with 100 nM microcystin-LR; gray bar, 10 μM β-carotene with 100 nM microcystin-LR; and black bar, 100 μM β-carotene with 100 nM microcystin-LR. Data represent the means ± SD of four determinants: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

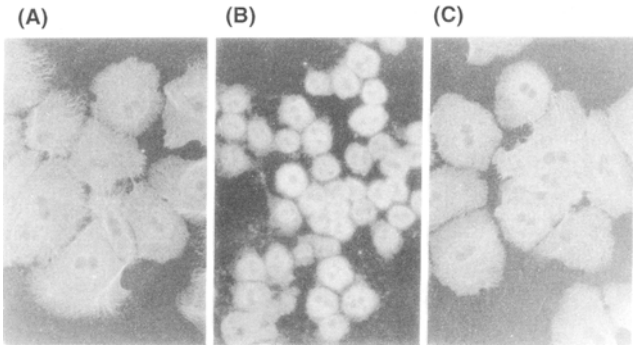


FIG. 6. Indirect immunofluorescence micrographs of cellular cytokeratin 18 in mouse primary cultured hepatocytes (×153). Cytokeratin 18 filaments in the hepatocytes (A) were retracted significantly by 100 nM Microcystin-LR (B). Cotreatment of 10 μM β-carotene protected the cytokeratin network against microcystin-LR (C). (A) Control; (B) 100 nM microcystin-LR; (C) 10 μM β-carotene with 100 nM microcystin-LR.

β-carotene protected the cytokeratin network in the hepatocytes from microcystin-induced disassembly (Fig. 6C).

DISCUSSION

In the present study, we have shown for the first time that several carotenoids antagonized okadaic acid class tumor promoters. Okadaic acid class tumor promoters are potent inhibitors of protein phosphatases 1 and 2A and induce hyperphosphorylation of cellular proteins (18,23,25–27). The intermediate filaments of cells are one of the targets of okadaic acid class tumor promoters, and hyperphosphorylation of the intermediate filaments causes the morphological changes of cells (25,26). All carotenoids tested herein protected mouse hepatocytes from the morphological changes induced by the tumor promoters and suppressed the tumor promoter-activated hyperphosphorylation of cytokeratins 8 and 18.

It was reported that cytokeratins 8 (55 kDa) and 18 (49 kDa), intermediate filaments of the primary cultured hepatocytes, are highly phosphorylated by microcystin-LR as compared to several other phosphorylated proteins in the hepatocytes (23). The 55 and 49 kDa bands appeared as major phosphorylated proteins after 3 h treatment with microcystin-LR (Fig. 4, lane 2); they were determined as cytokeratins 8 and 18, respectively, by immunoprecipitation (data not shown). The suppression of morphological changes of the hepatocytes by carotenoids may be a result of the marked reduction of cytokeratin hyperphosphorylation (Fig. 6). Phosphorylation of a specific subset of the many phosphorylation sites of these cytokeratins may disrupt the cellular cytokeratin network, thus resulting in morphological changes (23,28,29). β-Carotene may suppress hyperphosphorylation of the phosphorylation sites for the morphological changes of cells more specifically than other phosphorylation sites. Therefore, the suppression of the morphological changes seems more sensitive than the suppression of hyperphosphorylation of the other cellular proteins.

Suppression of hyperphosphorylation and the suppression of the morphological changes of the hepatocytes by

carotenoids are not clearly related to antioxidant or provitamin A activities . The emulsion of 10 μM β-carotene, astaxanthin, or esterified astaxanthin contained 48 μM α-tocopherol. However, the emulsion without carotenoids did not protect these cells against the morphological changes. Further, other carotenoids, such as canthaxanthin, which were dissolved in DMSO/THF (1:1, vol/vol) without α-tocopherol, also blocked the morphological changes of the hepatocytes, suggesting that α-tocopherol was not essential for the protective effect. α-Tocopherol is much more effective than β-carotene as an antioxidant for membrane lipids (30). Therefore, the antioxidant activity of β-carotene would not seem to play a significant role in its protection of the cells from the morphological changes caused by tumor promoters. In this study retinol, all-*trans* retinoic acid and 9-*cis* retinoic acid at the same concentrations as those of the carotenoids protected hepatocytes only weakly from morphological changes. These results indicated that suppression of morphological changes by carotenoids does not involve their conversion to retinoids.

Carotenoids strongly suppressed the microcystin-LR-induced morphological changes in primary culture mouse hepatocytes when added simultaneously with the tumor promoters. Carotenoids also suppressed the progression of morphological changes of the hepatocytes when β-carotene was added at 60 min after treatment with microcystin-LR (data not shown). These results indicated that carotenoids might not inhibit an uptake of microcystin-LR into the hepatocytes.

In the present study, we used 100 nM to 100 μM carotenoids. The concentration of β-carotene in human liver is reported to be 3–80 μmol/kg (28) (i.e., 3–80 μM if the specific gravity of the liver is estimated as L). Although it is hard to simply compare the intrahepatic concentrations of carotenoids between a human and a mouse or to estimate the real amount of β-carotene taken up by the hepatocytes in primary culture, the concentrations of carotenoids that suppressed the morphological changes may be within the physiological range.

Recently, it has been reported that β-carotene arrests tumor cells at G_0/G_1 or G_2 of the cell cycle (19,20,31,32) and that okadaic acid enhances cell cycling by inducing phosphorylation of cell cycle regulator proteins (33,34). Regulatory proteins of the cell cycle other than cyclins A and D, such as p53 and Rb antioncogene products, are also targets of okadaic acid class tumor promoters (35–37), and hyperphosphorylation of these antioncogene products is another important effect of this tumor promoter class. The β-carotene only affected the hyperphosphorylated intracellular proteins and did not reduce the basal protein phosphorylation levels of the control cells (Fig. 4). Therefore, the suppression of hyperphosphorylation of cellular proteins by the carotenoid might have some correlation with the arrest of the cell cycle.

Although the mechanisms by which carotenoids suppress the hyperphosphorylation of cellular proteins induced by microcystin-LR are not clearly understood and are now under investigation, the present results indicate further possibilities of the anticarcinogenic activities of carotenoids.

REFERENCES

1. Krinsky, N.I. (1991) Effects of Carotenoids in Cellular and Animal Systems, *Am. J. Clin. Nutr. 53*, 238S–246S.
2. Malone, W.F. (1991) Studies Evaluating Antioxidants and β-Carotene as Chemopreventives, *Atr.. J. Clin. Nutr. 53*, 305S–313S.
3. Stähelin, H.B., Gey, K.F., Eichholzer, M., and Lüdin, E. (1991) β-Carotene and Cancer Prevention: The Basal Study, *Am. J. Clin. Nutr. 53*, 265S–269S.
4. Krinsky, N.I. (1989) Carotenoids and Cancer in Animal Models, *J. Nutr. 119*, 123–126.
5. Peto, R., Doll, R., Buckley, J.D., and Sporn, M.B. (1981) Can Dietary Beta-Carotene Materially Reduce Human Cancer Rates?, *Nature 290*, 201–209.
6. Alabaster, O., Tang, Z.C., Frost, A., and Slivapurkar, N. (1995) Effect of β-Carotene and Wheat Bran Fiber on Colonic Aberrant Crypt and Tumor Formation in Rats Exposed to Azoxymethane and High Dietary Fat, *Carcinogenesis 16*, 127–132.
7. Teicher, B.A., Schwartz, J.L., Holden, S.A., Ara, G., and Northey, D. (1994) *In vivo* Modulation of Several Anticancer Agents by β-Carotene, *Cancer Chemother. Pharmacol. 34*, 235–241.
8. Brock, K.E., Berry, G., Mock, P.A., McLennan, R., Truswell, A.S., and Brinton, L.A. (1988) Nutrients in Diet and Plasma and Risk of *in situ* Cervical Cancer, *J. Natl. Comer. Inst. 80*, 580–585.
9. Connett, J.E., Kuller, L.H., Kjelsberg, M.O., Polk, B.F., Colins, G., Rider, A., and Hulley, S.B. (1989) Relationship Between Carotenoids and Cancer—The Multiple Risk Factor Intervention Trial (MRFIT) Study, *Cancer 64*, 126–134.
10. Menskes, M.S., Comstock, G.W., Vuilleumier, J.P., Helsing, K.J., Rider, A.A., and Brookmeyer, R. (1986) Serum Beta-Carotene, Vitamins A and E, Selenium, and the Risk of Lung Cancer, *N. Engl. J. Med. 315*, 1250–1254.
11. Blot, W.J., Li, J.-Y., Taylor, P.R., Guo, W., Dawsey, S., Wang, G.-Q., Yang, C.S., Zheng, S.-F., Gail, M., Li, G.-Y., Yu, Y., Liu, B., Tangrea, J., Sun, Y., Liu, F., Fraumeni, J.F. Jr., Zhang, Y.-H., and Li, B. (1993) Nutrition Intervention Trials in Linxian, China: Supplementation with Specific Vitamin/Mineral Combinations, Cancer Incidence, and Disease-Specific Mortality in the General Population, *J. Natl. Cancer Inst. 85*, 1483–1492.
12. Scita, G., and Wolf, G. (1994) Retinoic Acid and Beta-Carotene Inhibit Fibronectin Synthesis and Release by Fibroblasts; Antagonism to Phorbol Ester, *Carcinogenesis 15*, 1043–1048.
13. The ATBC Cancer Prevention Study Group (1994) The Alpha-Tocopherol, Beta-Carotene Lung Cancer Prevention Study: Design, Methods, Participant Characteristics, and Compliance, *Ann. Epidemiol. 4*, 1–10.
14. Potischman N., McCulloch, C.E., Byers, T., Nemoto, T., Stubbe, N., Milch R., Parker, R., Rasmussen, K.M., Root, M., Graham, S., and Campbell T.C. (1990) Breast Cancer and Dietary and Plasma Concentrations of Carotenoids and Vitamin A, *Am. J. Clin. Nutr. 52*, 909–915.
15. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura T. (1988) Okadaic Acid: An Additional Non-Phorbol-12-Tetradecanoate-13-Acetate-Type Tumor Promoter, *Proc. Natl. Acad. Sci USA. 85*, 1768–1771.
16. Fujiki, H., and Suganuma, M.(1993) Tumor Promotion by Inhibitors of Protein Phosphatases 1 and 2A: The Okadaic Acid Class of Compounds, *Adv. in Can. Res. 61*, 143–194.
17. Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., and Fujiki, H. (1992) Liver Tumor Promotion by the Cyanobacterial Cyclic

Peptide Toxin Microcystin-LR, *J. Cancer Res. Clin. Oncol. 118*, 420–424.
18. Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K., Ichihara, A., Carmichael, W.W., and Fujiki, H. (1990) Inhibition of Protein Phosphatases by Microcystin and Nodularin Associated with Hepatotoxicity, *J. Cancer Res. Clin. Oncol. 116*, 609–614.
19. Schwartz, J.L., Antoniades, D.Z., and Zhao, S. (1993) Molecular and Biochemical Reprogramming of Oncogenesis Through the Activity of Prooxidants and Antioxidants, *Ann. N.Y. Acad. Sci. 686*, 262–278.
20. Schwartz, J.L. (1994) *In Vitro* Biological Methods for Determination of Carotenoid Activity, *Methods in Enzymol. 214*, 226–256.
21. Schwartz, J.L., Singh, R.P., Teicher, B., Wright, J.E., Frites, D.M., and Shklar, G. (1990) Induction of a 70kD Protein Associated with the Selective Cytotoxicity of Beta-Carotene in Human Epidermal Carcinoma, *Biochem. Biophys. Res. Commun. 169*, 941–946.
22. Schwartz, J.L., Tanaka, J., Khandekar, V., Herman, T.S., and Teicher, B.A. (1992) β-Carotene and/or Vitamin E as Modulators of Alkylating Agents in SCC-25 Human Squamous Carcinoma Cells, *Cancer Chemother. Pharmacol. 29*, 207–213.
23. Ohta, T., Nishiwaki, R., Yatsunami, J., Komori, A., Suganuma, M., and Fujiki, H. (1992) Hyperphosphorylation of Cytokeratin 8 and 18 by Microcytin-LR, a New Liver Tumor Promoter, in Primary Cultured Rat Hepatocytes, *Carcinogenesis 13*, 2443–2447.
24. Laemmli, U.K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, *Nature 227*, 680–685.
25. Yatsunami, J., Komori, A., Ohta, T., Suganuma, M., Yuspa, S.H., and Fujiki, H. (1993) Hyperphosphorylation of Cytokeratins by Okadaic Acid Class Tumor Promoters in Primary Human Keratinocytes, *Cancer Res. 53*, 992–996.
26. Yatsunami, J., Fujiki, H., Suganuma, M., Yoshizawa, S., Eriksson, J.E., Olson, M.O.J., and Goldman, R.D. (1991) Vimentin is Hyperphosphorylated in Primary Human Fibroblasts Treated with Okadaic Acid, *Biochem. Biophys. Res. Commun. 107*, 1165–1170.
27. Ohta, T., Sueoka, E., Iida, N., Komori, A., Suganuma, M., Nishiwaki, R., Tatematsu, M., Kim, S.-J., Carmichael, W.W., and Fujiki, H. (1994) Nodularin, A Potent Inhibitor of Protein Phosphatases 1 and 2A, Is a New Environmental Carcinogen in Male F344 Rat Liver, *Cancer Res. 54*, 6402–6406.
28. Schmitz, H.H., Poor, C.L., Wellman, R.B., and Erdman, J.W., Jr. (1991) Concentrations of Selected Carotenoids and Vitamin A in Human Liver, Kidney and Lung Tissue, *J. Nutr. 121*, 1613–1621.
29. Fuchs, E., and Weber, K. (1994) Intermediate Filaments: Structure, Dynamics, Function, and Disease, *Annu. Rev. Biochem. 63*, 345–382.
30. Palozza, P., and Krinsky, N.I. (1992) Antioxidant Effects of Carotenoids *in vivo* and *in vitro*: An Overview, *Methods in Enzymology 213*, 403–429.
31. Schwartz, J.L., Teicher, B.A., and Stringham, J.M. (1992) Carotenoids Cell Cycle Specific Inhibitors, *Cancer Res. 33*, 441(a).
32. Schwartz, J.L. (1993) β-Carotene Induced Programmed Cell Death, *J. Dent. Res. 72*, 285.
33. Ishida, Y., Furukawa, Y., de Caprio, J.A., Saito, M., and Griffin, J.D. (1992) Treatment of Myeloid Leukemic Cells with the Phosphatase Inhibitor Okadaic Acid Induces Cell Cycle Arrest at Either G1/S or G2/M Depending on Dose, *J. Cellular Physiol. 150*, 484–492.
34. Van Dolah, F.M., and Ramsdell, J.S (1992) Okadaic Acid Inhibits a Protein Phosphatase Activity Involved in Formation of the Mitotic Spindle of GH_4 Rat Pituitary Cells, *J. Cellular Physiol. 152*, 190–198.
35. Yatsunami, J., Komori, A., Ohta, T., Suganuma, M., and Fujiki, H. (1993) Hyperphosphorylation of Retinoblastoma Protein and p53 by Okadaic Acid, a Tumor Promoter, *Cancer Res. 53*, 239–241.
36. Zhang, W., McClain, C., Gau, J., Guo, X.D., and Deisseroth, A.B. (1994) Hyperphosphorylation of p53 Induced by Okadaic Acid Attenuates Its Transcriptional Activation Function, *Cancer Res. 54*, 4448–4453.
37. Fuchs, B., Hecker, D., and Scheidtmann, K.H. (1995) Phosphorylation Studies on Rat p53 Using the Baculovirus Expression System—Manipulation of the Phosphorylation State with Okadaic Acid and Influence on DNA Binding, *Eur. J. Biochem. 228*, 625–639.

[Received June 9, 1995, and in final revised form September 15, 1995; Revision accepted September 18, 1995]

Dietary Menhaden Oil Enhances Mitomycin C Antitumor Activity Toward Human Mammary Carcinoma MX-1

Yu Shao, Lani Pardini and Ronald S. Pardini*

Allie M. Lee Laboratory for Cancer Research, Department of Biochemistry, University of Nevada, Reno, Nevada 89557

ABSTRACT: In the present study, we investigated the effects of high levels of dietary fish oil on the growth of MX-1 human mammary carcinoma and its response to mitomycin C (MC) treatment in athymic mice. We found that high levels of dietary fish oil (20% menhaden oil + 5% corn oil, w/w) compared to a control diet (5% corn oil, w/w) not only lowered the tumor growth rate, but also increased the tumor response to MC treatment. We also found that high levels of dietary fish oil significantly increased the activities of tumor xanthine oxidase and DT-diaphorase, which are proposed to be involved in the bioreductive activation of MC. Since menhaden oil is highly unsaturated, its intake caused a significant increase in the degree of fatty acid unsaturation in tumor membrane phospholipids. This alteration in tumor membrane phospholipids made the tumor more susceptible to oxidative stress, as indicated by the increased levels of both endogenous lipid peroxidation and protein oxidation after feeding the host animals the menhaden oil diet. In addition, the tumor antioxidant enzyme activities, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPOx), and glutathione S-transferase peroxidase (GSTPx), were all significantly enhanced by feeding a diet high in fish oil. MC treatment caused further increases in tumor lipid peroxidation and protein oxidation, as well as in the activities of CAT, SOD, GPOx, and GSTPx, suggesting that MC causes oxidative stress in this tumor model which is exacerbated by feeding a diet high in menhaden oil. Thus, feeding a diet rich in menhaden oil decreased the growth of human mammary carcinoma MX-1, increased its responsiveness to MC, and increased its susceptibility to endogenous and MC-induced oxidative stress, and increased the tumor activities of two enzymes proposed to be involved in the bioactivation of MC, that is, DT-diaphorase and xanthine oxidase. These findings support a role of these two enzymes in the bioactivating of MC and indicate that the type of dietary fat may be important in tumor response to therapy.
Lipids 30, 1035–1045 (1995).

Fish oil, which contains high levels of n-3 long-chain polyunsaturated fatty acids (PUFAs), has been shown to suppress mammary gland tumorigenesis in rodent models (1–4). Several hypotheses have been proposed to explain how fish oil inhibits mammary tumorigenic processes, including inhibition and/or alteration of eicosanoid metabolism, induction of oxidative stress, and a deficiency in essential fatty acids in fish oil diets (1–4). The n-3 long-chain PUFAs contained in fish oil mainly are eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA), which contain five and six double bonds, respectively. When the PUFAs are presented in the diets, they are readily incorporated into tumor membrane phospholipids (1–4), resulting in an increase in the level of unsaturation of the membrane lipids. This makes the membrane more susceptible to peroxidation *in vitro* and *in vivo*, which leads to an increased accumulation of cytostatic and/or cytotoxic lipid peroxidation products, especially in the tumor (5,6).

Mitomycin C (MC), a quinone-containing antibiotic active in the treatment of solid tumors, undergoes both one-electron and two-electron reduction by the MC bioreductive activating enzymes (7–10). The two-electron reduction of MC forms a hydroquinone structure which can cross-link DNA, especially under hypoxic conditions. The one-electron reduction of MC results in the formation of the semiquinone free radical, which can interact with molecular oxygen to form toxic reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxy radical (•OH). The free radical dependent process could be critical to the antitumor activity of MC, for either DNA alkylation, DNA oxidation, or peroxidation of cellular membrane phospholipids (7–10).

It has been reported that high levels of dietary fat enhance the activities of a variety of enzymes, including those responsible for metabolizing xenobiotics (11–13). In a previous study (14), we found that MC bioreductive activating enzymes, including DT-diaphorase (DTD) and cytochrome b_5 reductase (CB5R) in human mammary carcinoma MX-1 heterotransplanted in the nude mice, were increased after feeding the host animals a high corn oil (CO) diet (25% CO, w/w). This would result in an increase in the formation of one-electron or two-electron reductive metabolites of MC in the tumor, increasing its antitumor effect through either increasing DNA cross-linking or production of toxic free radicals. In addition, we observed that feeding a high CO diet caused an

*To whom correspondence should be addressed at Department of Biochemistry, Mail Stop 330, University of Nevada, Reno, NV 89557.

Abbreviations: CAT, catalase; CB5R, cytochrome b_5 reductase; CCR, cytochrome C reductase; CO, corn oil; DHA, docosahexaenoic acid; DNPH, 3,4-dinitrophenyl hydrazine; DTD, DT-diaphorase; EPA, eicosapentaenoic acid; GPOx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; GSTPx, glutathione S-transferase peroxidase; HNE, 4-hydroxy-nonenal; MC, mitomycin C; MO, menhaden oil; PL, phospholipid; PUFAs, polyunsaturated fatty acids; SOD, superoxide dismutase; TBARS, thiobarbituric reactive substances; T/C, treat/control ratio; XD, xanthine dehydrogenase; XO, xanthine oxidase.

Copyright © 1995 by AOCS Press

increased tumor response to MC treatment and increased oxidative stress after MC treatment, as indicated by elevated lipid peroxidation and enhanced antioxidant enzyme activities in the tumor.

In the present study, we determined the effect of feeding a diet high in fish (menhaden) oil [20% menhaden oil (MO) + 5% CO) on the growth, susceptibility to MC treatment, endogenous and MC-induced oxidative stress, and effect on MC-activating enzyme activities in human mammary carcinoma MX-1 grown in athymic mice.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. The BCA total protein assay kit was obtained from Pierce Co. (Rockford, IL). Dietary components were purchased from Dyets Inc. (Bethlehem, PA).

Animals and diets. Forty-eight female adult athymic mice (female heterozygous BALB/c nu/+) were used in the present studies. The mice were housed under aseptic conditions (germ-free laminar-flow hood, sterilized cages, bedding, and water) at 27°C. Autoclaved laboratory mouse chow (Dyets Inc.) was fed *ad libitum* before the experimental diet was fed. The experimental diet was composed of the casein-based, semipurified AIN-76A diet (15,16) with some modifications according to Birt *et al.* (17), containing 20% MO + 5% CO (w/w) or 5% CO (w/w) (Table 1).

Tumor implantation and MC treatment. The animals were assigned randomly to the high-MO or low-CO diet, with 24 mice in each of the two experimental groups. Mice were fed their respective experimental diets for 20 d prior to tumor inoculation. A tumor fragment (1–2 mm^3) of human mammary carcinoma MX-1 was implanted subcutaneously on the right foreflank of each nude mouse. Tumor weights were recorded every 3–4 d and were estimated by the calculation based on the formula:

$$\text{tumor weight (mg)} = \frac{A \cdot B \cdot C}{2} \quad [1]$$

where A, B, and C represent the three perpendicular diameters of the tumor in millimeters (18). The percentage T/C ratio was calculated by the mean tumor weight of the treated group divided by the mean tumor weight of the control group (MC untreated) multiplied by 100 (18). All the mice were assigned to the following two experiments.

Experiment 1. Twelve mice in each dietary group with a total of 24 were used in this experiment. Ten days after tumor implantation, half of the mice in high MO and low CO groups (six animals in each group) were administrated with MC at the dosage of 0.5 mg/kg body weight, i.p., once a week for four consecutive weeks. Thirty days after tumor implantation, the animals in both dietary groups that did not receive MC treatment were sacrificed by cervical dislocation due to the large tumor volume. The remaining animals (MC treated) were kept on the experimental diets for another 30 d, and tumor volume and body weights were recorded as above. (Their tumors were too small to conduct biochemical testing.)

Experiment 2. Another 24 mice with 12 in each dietary group were used. Half (six animals) of the mice in each group received MC treatment 15 d after tumor implantation for three weeks, with the same dosage as in experiment 1. All the mice were sacrificed 24 h after the third MC treatment. Tumors were removed immediately, either homogenized in 3 mL of ice-cold 50 mM phosphate buffer (pH 7.0) for enzyme activities, lipid peroxidation, and protein oxidation assays, or kept in liquid N_2 for glutathione content and membrane phospholipid composition measurement. The homogenate was centrifugated at 10,000 × *g* for 30 min and the supernatant was retained for enzymatic assays.

Enzyme assays. DTD activity was measured as the dicumarol-sensitive reduction of 2,6-dichlorophenolindophenol, according to the method of Ernster (19) and modified by Benson *et al.* (20). Xanthine oxidase/dehydrogenase activities were assayed with the formation of uric acid from xanthine measured spectrophotometrically at 293 nm (21). Cytochrome C reductase (CCR) activity was measured according to the method of Masters *et al.* (22) and Yasukochi and Masters (23). Superoxide dismutase (SOD) activity was measured using the method described by Oberely and Spitz (24). Catalase (CAT) activity was determined by measuring the sample catalyzed disappearance of H_2O_2 spectrometrically at 240 nm (25). Glutathione peroxidase (GPOx) activity was measured in a coupled enzyme reaction with glutathione reductase (GR) (26). Glutathione S-transferase peroxidase (GSTPx) activity was measured similar to GPOx activity (27). GR activity was measured as described by Racker (28).

TABLE 1
Composition of Experimental Diets[a]

Ingredient (g)	Control (CO)	Menhaden oil (MO)
Corn oil (CO)	5	5
MO	0	20
Casein	20	24.6
DL-methionine	0.3	0.5
Cornstarch	15	9
Sucrose	50	29.1
Cellulose	5	6
AIN vitamin mix	1	1.2
AIN mineral mix	3.5	4.3
Choline bitartrate	0.2	0.3
Total weight	100	100
Caloric density (kcal/g)[b]	3.85	4.85
% of calories from fat	11.69	46.39
Unsaturation index	141	243
Daily diet consumed (g/mouse)[c]	4.36 ± 0.89	3.54 ± 0.80
Daily caloric intake (kcal/mouse)	16.79 ± 3.43	17.17 ± 3.88

[a]Formulated in accordance with the American Institute of Nutrition recommendation (15,16) and modified according to Birt *et al.* (17).
[b]Calculated as 4.0 kcal/g for starch, sucrose, and casein, and 9.0 kcal/g for corn oil and menhaden oil.
[c]Calculated daily by subtracting the amount of uneaten food from the amount of the food added the day before.

GST activity was determined by the method according to Mannervik and Guthenberg (29).

Lipid peroxidation. Malondialdehyde (MDA) was measured as an indication of lipid peroxidation by the method of Buege and Aust (30). Thiobarbituric acid [0.375% (wt/vol) with 15% trichloroacetic acid and 0.25 N HCl] was mixed with the test sample and heated in boiling water for 10 min. The precipitate was removed by centrifugation at 1000 × *g* for 10 min and the supernatant was removed for further analysis. Thiobarbituric reactive substances (TBARS) in the supernatant were measured spectrophotometrically at 535 nm.

Protein oxidation. Protein carbonyl groups were measured as an indication of protein oxidation by the method according to Levine *et al.* (31). Schiff's bases was also measured by this procedure. DNA was removed from the fraction by streptomycin precipitation. The remaining protein was precipitated and the pellet mixed with 10 mM 3,4-dinitrophenyl hydrazine (DNPH) for 1 h. Excess DNPH is removed with 1:1 ethanol/ethyl acetate mixture and the pellet is redissolved in 0.6 M guanidine in 20 mM potassium phosphate buffer, pH 6.3. Solution absorbance was then measured at 360–390 nm ($\varepsilon 360–390 = 22{,}000\ M^{-1}cm^{-1}$) to determine the protein carbonyl content. Control sample absorbance without DNPH was subtracted from the test absorbance.

Tumor glutathione (GSH) levels. Tumors were homogenized in 10% (vol/vol) perchloric acid containing 1 mM bathophenanthrolinedisulfonic acid. This acid extract was then spun at 15,000 × *g* for 15 min, and the supernatant derivatized and analyzed by HPLC by the method of Fariss and Reed (32). HPLC analysis was carried out using a Beckman 338 binary gradient system (Beckman Instruments, Palo Alto, CA) fitted with an a-Aminopropyl-Spherisorb column (Custom LC, Inc., Houston, TX).

Membrane lipid content. Tumor membrane lipid compositions were analyzed as previously described (18). Lipid was extracted from tumor membrane with chloroform/methanol (2:1, vol/vol). Fatty acid methyl esters were obtained by transesterification with borontrifluoride/methanol reagent. The methyl esters were analyzed by gas chromatography.

Statistical analysis. Data are expressed as means ± SD with n = 6 per group; differences in means were assessed by analysis of variance using the SAS general linear models program. Group means were considered to be significantly different at $P < 0.05$.

RESULTS

Tumor growth studies. Dietary intake of high levels of n-3 PUFAs has been reported to inhibit tumor growth and tumorigenesis in rodent models (1–4). In this study, we employed a diet high in n-3 PUFAs to evaluate its effects on tumor growth and responsiveness to MC chemotherapy. As shown in Table 2, the growth of human mammary carcinoma MX-1 was significantly inhibited in the animals fed a diet high in MO in Experiment 1. At the 30th day after tumor implantation, the average tumor weight in the high-MO group was 2016 ± 875 mg, whereas in the low-CO group it was 3500 ± 1050 mg (43% inhibition by MO). The treatment/control (T/C) ratios resulting from MC treatment were 1 for MO group and 10 for CO group with final mean tumor weights being 15 and 342 mg, respectively. Because 50 and 17% of the animals were clear of tumor at 30 d, the average tumor size of the remaining tumors at day 30 were 29 ± 10 mg for MO and 386 ± 102 mg for CO (Table 2). The MO-fed group possessed tenfold better response to MC compared to the CO-fed group. These T/C ratios were calculated by comparing the MC-treated group to its respective dietary control (see Materials and Methods section). If we compared the MO-fed MC-treated tumor to the CO control (dual effects from dietary MO feeding and MC treatment), the combined T/C ratio becomes 0.4. Thus feeding a 25% MO diet resulted in a dramatic reduction in tumor size following MC treatment compared to the 5% CO control. The animals

TABLE 2
Antineoplastic Activity of MC Against MX-1 After Different Diet Feeding

Dietary group	Treatment	Mean tumor weight (mg ± SD) (day 30)	T/C ratio day 30[a]	Combined T/C ratio day 30[b]	% Clear[c] Day 30	% Clear[c] Day 60	No. of animals used (N)	Net change in body weight between day 0 and day 30 (g ± SD)[d]
CO (5% corn oil)	Control	3500 ± 1050			0	—	6	5.1 ± 1.2
			10	10				
	MC	342 ± 164[e]			17	0	6	2.1 ± 0.4
MO	Control	2016 ± 875[f]			0	—	6	4.5 ± 1.0
(5% corn oil			1	0.43				
+ 20% menhaden oil)	MC	15 ± 17[e,g]			50	33	6	1.5 ± 0.3

[a]The percentage treatment/control ratio (T/C ratio) was calculated by taking the mean tumor weight of the mitomycin C (MC)-treated group divided by the mean tumor weight of the respective control (MC untreated) multiplied by 100. Other abbreviations as in Table 1.
[b]The combined T/C ratio accounts for the effect of diet and MC treatment and is calculated by taking the mean tumor weight of the MC-treated groups divided by the mean tumor weight of the CO control group multiplied by 100.
[c]Percent of animals free of visible or palpable tumor at the days indicated (the days after tumor implantation).
[d]Net change in body weight from day 0 (day of tumor implantation) to day 30 (day of termination of experiment).
[e]The average size of the tumors in mice that had visible or palpable tumors were 386 ± 102 mg in CO group and 29 ± 10 mg in MO group.
[f]Mean tumor weight is statistically different from the CO control group, $P < 0.05$.
[g]Mean tumor weight is statistically different from the CO+MC group, $P < 0.01$.

were maintained on the experimental diets for an additional 30 d following MC treatment, and observed for reoccurring tumor. In the low CO-fed group, the mouse free of tumor at day 30 experienced reoccurrence by the 60th day following tumor implantation. Of the three tumor-free mice in the MO group, only one appeared to have tumor regrowth, resulting in 2 out of 6 mice free of visible or palpable tumor (33% tumor-free) at the 60th day following tumor implantation. These data clearly indicate that the intake of high levels of MO not only significantly inhibited human mammary carcinoma MX-1 growth, but significantly enhanced the tumor responsiveness to MC treatment.

In Experiment 2, the same number of animals and same experimental diets were used. The only difference between these two experiments was that the time of the first MC treatment was postponed from the 10th day to the 15th day after tumor implantation in order to obtain large tumors to provide sufficient mass to measure tumor lipid peroxidation, protein oxidation, antioxidant enzyme activities, and MC-activating enzyme activities in the tumor. The results of this second study were similar to those from Experiment 1 (Table 2) in that the tumor growth was significantly suppressed by feeding MO diet and the tumor response to MC treatment was enhanced markedly by the MO diet. At the end of the second experiment (26 d after tumor implantation), the T/C ratios were 5 for MO group and 17 for CO group, indicating that the tumors from the MO-fed group were more sensitive to MC than the CO-fed group. There were no differences in host body weights between different dietary groups and MC treatment.

Tumor enzyme activities. Figure 1 shows the alterations of tumor MC bioreductive-activating enzyme activities by the different diets and MC treatment. In the present experiment, DTD activity was increased significantly by MO feeding ($P < 0.001$) and further increased by MC treatment ($P < 0.05$; Fig. 1A) compared to CO feeding. MC treatment had no effect on DTD activity in the CO group (Fig. 1A). CB5R activity was not affected by the diet alone. After MC treatment, CB5R activity was increased in the MO group (MO + MC group, $P < 0.01$; Fig. 1B) compared to the MO, CO + MC or CO groups. CCR activity was not altered by diet alone either, but was increased by MC treatment in both dietary groups ($P < 0.01$, Fig. 1C). A similar pattern was observed for xanthine dehydrogenase (XD) as CCR (Fig. 1D). Xanthine oxidase (XO) was significantly increased by feeding a diet high in MO ($P < 0.05$) and further increased by MC treatment (Fig. 1E) in both dietary groups. These results indicate that MO feeding and/or MC treatment changed tumor drug activating enzyme activities which are probably responsible for the enhanced antineoplastic effect of MC observed after feeding a high-MO diet.

Feeding MO which is rich in n-3 long-chain PUFAs will increase tumor membrane phospholipids (PLs) unsaturation by incorporating these highly unsaturated fatty acids into PLs (1–4). Figure 2 shows that dietary menhaden oil and MC treatment significantly altered tumor antioxidant enzyme activities. GPOx activity was increased by a high–n-3 PUFAs diet (MO, $P < 0.05$) and further increased by MC treatment ($P < 0.001$ in MO and $P < 0.05$ in CO). Similar changes were observed for GSTPx activity (Fig. 2C). Both tumor CAT and SOD activities were elevated after feeding MO and further elevated by MC treatment (Figs. 2E and 2F). As we observed in a previous study (14), GST activity was not changed by either dietary MO or MC treatment (Fig. 2B). GR activity was inhibited by MC treatment, but increased by feeding the high-MO diet ($P < 0.01$; Fig. 2D). These findings, along with tumor lipid peroxidation and protein oxidation data (discussed later), suggest that a high intake of n-3 PUFAs (MO in this study) causes an increase in endogenous oxidative stress in the tumor, presumably by changing the unsaturated fatty acid composition of tumor membrane lipids. This could be responsible for the observed suppressed tumor growth and enhanced the response to the prooxidant antitumor agents such as MC.

Tumor GSH. Tumor levels of GSH were measured after feeding the host animals with different diets, and the results are shown in Table 3. Tumor GSH levels were decreased by feeding the PUFA-rich MO diet ($P < 0.05$). Although the glutathione disulfide (GSSG) levels were not changed in the two different dietary groups, the GSH/GSSG ratio was significantly decreased in the MO group. These data indicate that feeding a diet rich in high levels of unsaturated PUFAs (such as MO) causes a decrease of GSH level in the tumor, which is consistent with increased tumor oxidative stress, and increased response to chemotherapy.

Lipid peroxidation and protein oxidation. The data in Figure 3 demonstrate that after feeding an MO-rich diet, a significant increase in tumor lipid peroxidation and protein oxidation occurred. MC treatment further increased both tumor lipid peroxidation and protein oxidation, similar to the responses of the antioxidant enzymes. Again, these results indicate that both dietary menhaden oil and MC treatment caused oxidative stress in this tumor model. These results are consistent with the observed decreased tumor growth and increased responsiveness to MC treatment.

Tumor fatty acid composition. As shown in Figure 4, tumor membrane PL fatty acid composition was changed by the different diets. There was a significant elevation of 18:1n-9, 18:2n-6, and 20:4n-6 fatty acids in the tumor membranes from the CO group, whereas in the tumor membranes from the MO group, there were significant increases of n-3 PUFAs such as 18:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. This change in the fatty acid pattern reflects an increased degree of unsaturation, with an unsaturation index of 208 in the MO-fed groups compared to 147 in the CO-fed groups, and, therefore, susceptibility to oxidative stress in the tumor lipid from the MO-fed group compared to the CO-fed group (Fig. 4).

DISCUSSION

Fish oil has been reported to inhibit mammary tumorigenesis in a variety of animal models, including carcinogen-induced rat and mouse models, transplantable mammary tumor mod-

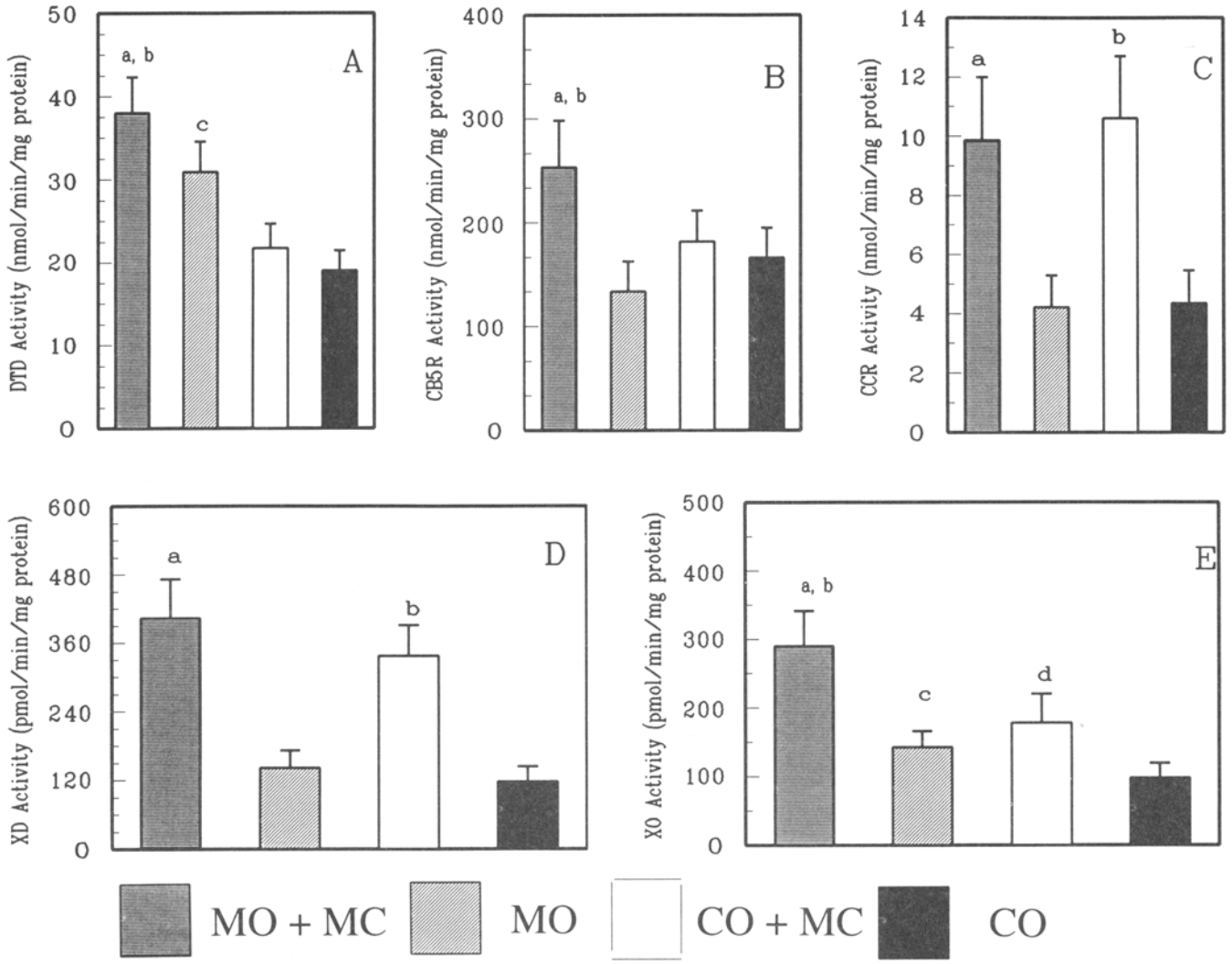


FIG. 1. Effects of feeding a diet high in menhaden oil (MO), or low in corn oil (CO) and mitomycin C (MC) treatment on tumor (A) DT-diaphorase (DTD), (B) cytochrome b_5 reductase (CB5R), (C) cytochrome C reductase (CCR), (D) xanthine dehydrogenase (XD), and (E) xanthine oxidase (XO) activities. Statistically significant differences were in (A) DTD activity between MO + MC and MO groups (a: $P < 0.05$), MO + MC and CO + MC groups (b: $P < 0.001$), and between MO and CO groups (c: $P < 0.001$); in (B) CB5R activity between MO + MC and MO groups (a: $P < 0.01$), between MO + MC and CO + MC groups (b: $P < 0.01$); (C) CCR activity between MO + MC and MO groups (a: $P < 0.01$) and between CO + MC and CO groups (b: $P < 0.01$); (D) in xanthine dehydrogenase activity between MO + MC and MO groups (a: $P < 0.01$) and between CO + MC and CO groups (b: $P < 0.01$); and (E) in xanthine oxidase activity between MO + MC and MO groups (a: $P < 0.01$), between MO + MC and CO + MC groups (b: $P < 0.01$), between MO and CO groups (c: $P < 0.05$), and between CO + MC and CO groups (d: $P < 0.05$).

els, or spontaneous mouse mammary tumor models (for review, see Refs. 1–4). Because fish oils are often deficient or marginal in the essential fatty acids, such as linoleic acid (18:2n-6), it is possible that the suppression of mammary tumorigenesis exerted by a high–fish oil diet may be due to insufficient amounts of linoleic acid. In fact, supplementation of fish oil diet with amounts of linoleic acid has been reported to block or partially block the inhibitory effects on mammary tumor development by a high–fish oil diet (33–36). In this study, we supplemented the high–fish oil diet with 5% corn oil, to provide essential fatty acid as recommended by the American Institute of Nutrition (15,16). We also observed a significant inhibitory effect on the growth of human mammary carcinoma by feeding a diet high in MO compared to a low-CO diet (Table 2). Thus, the inhibitory effect of MO on human mammary carcinoma growth in this study should not be due to the lack of sufficient essential fatty acids in the MO diet.

Previous studies have shown that dietary fat influences membrane structure and function (1–4) and have also demonstrated that membranes with a high unsaturation index are more sensitive to peroxidation (37–40). In this study, MO feeding resulted in a higher amount of n-3 PUFAs being incorporated into tumor membrane PLs with a decreased incorporation of arachidonic acid (20:4n-6; Fig. 4). These long-chain PUFAs significantly increased the degree of tumor membrane lipid unsaturation compared to the CO group, with a calculated unsaturation index of 208 and 147, respectively.

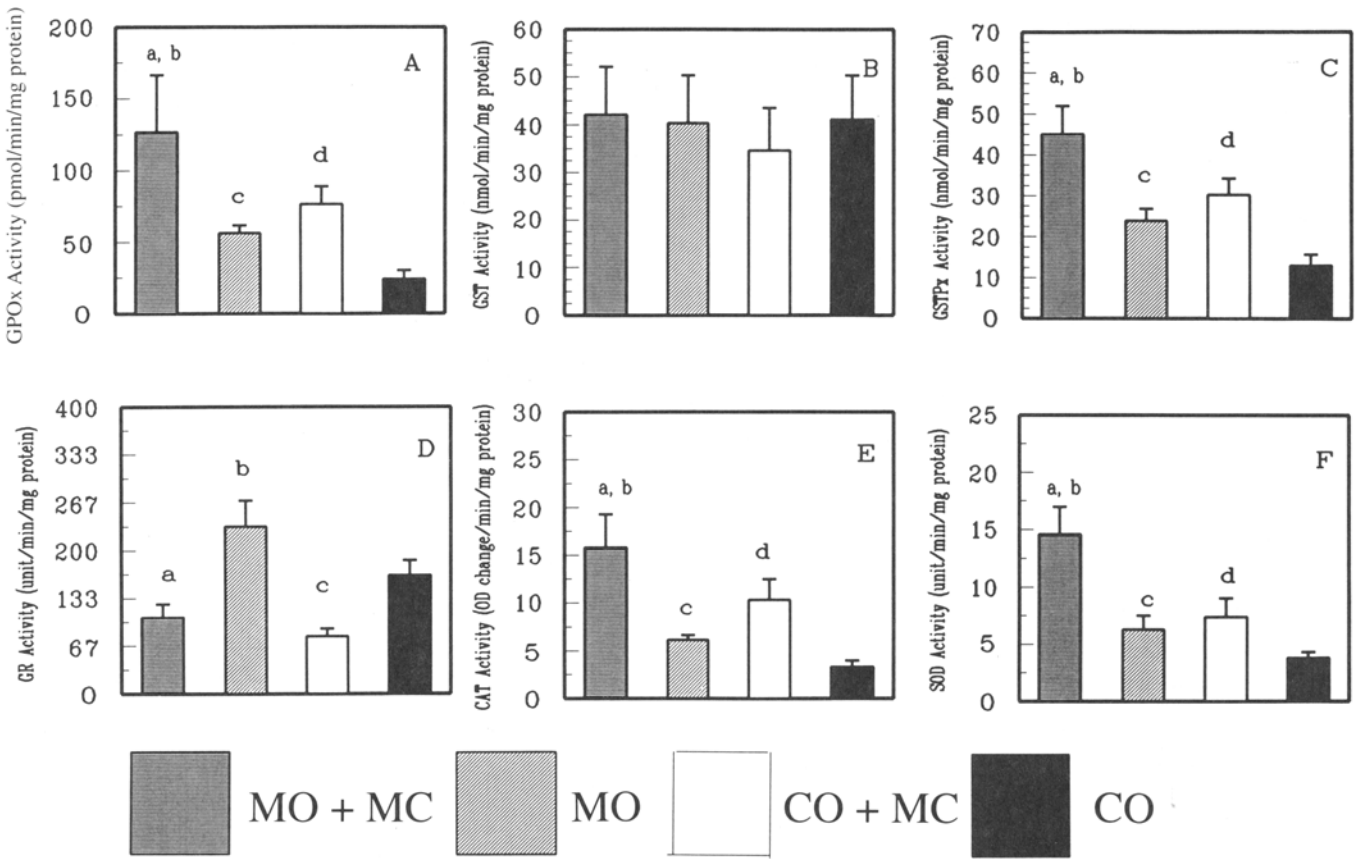


FIG. 2. Effects of feeding a diet high in MO, or low in CO and MC treatment on tumor (A) glutathione peroxidase (GPOx), (B) glutathione S-transferase (GST), (C) glutathione S-transferase peroxidase (GSTPx), (D) glutathione reductase (GR), (E) catalase (CAT), and (F) superoxide dismutase (SOD) activities. Statistically significant differences were (A) GPOx activity between MO + MC and MO groups (a: $P < 0.001$), between MO + MC and CO + MC groups (b: $P < 0.05$), between MO and CO groups (c: $P < 0.05$), and between CO + MC and CO groups (d: $P < 0.05$); (C) in GSTPx activity between MO + MC and MO groups (a: $P < 0.001$), between MO + MC and CO + MC groups (b: $P < 0.01$), between MO and CO groups (c: $P < 0.05$), and between CO + MC and CO groups (d: $P < 0.01$); (D) in GR activity between MO + MC and MO groups (a: $P < 0.01$), between MO and CO groups (b: $P < 0.01$), and between CO + MC and CO groups (c: $P < 0.01$); (E) in CAT activity between MO + MC and MO groups (a: $P < 0.001$), between MO + MC and CO + MC groups (b: $P < 0.01$), between MO and CO groups (c: $P < 0.05$), and between CO + MC and CO groups (d: $P < 0.01$); and (F) in SOD activity between MO + MC and MO groups (a: $P < 0.001$), between MO + MC and CO + MC groups (b: $P < 0.01$), between MO and CO groups (c: $P < 0.05$), and between CO + MC and CO groups (d: $P < 0.01$). Abbreviation as in Figure 1.

This increased level of unsaturation in the MO group increased the tumor lipid susceptibility to lipid peroxidation and subsequent endogeneous oxidative stress, as demonstrated by the following indicators of oxidative stress. (i) A significant elevation in the amounts of lipid peroxidation products (TBARS) and protein oxidation were observed in the tumor from the MO-fed group (Fig. 3). (ii) Antioxidant enzymes which participate in cellular protection against lipid peroxidation such as SOD, CAT, GPOx, and GSTPx were also activated by MO feeding (Fig. 2). (iii) Tumor GSH levels were

TABLE 3
Glutathione and Glutathione Disulfide Status in Human Mammary Carcinoma MX-1[a]

Dietary group	n[b]	GSH	GSSG	GSH/GSSG
CO (5% CO)	6	2.20 ± 0.13	0.10 ± 0.01	22.0 ± 0.5
MO (5% CO + 20% MO)	6	1.40 ± 0.02[c]	0.10 ± 0.01	14.0 ± 0.8[c]

[a]Values are means ± SD in μmol/g wet tumor wt for glutathione (GSH) and glutathione disulfide (GSSG). Other abbreviations as in Table 1.
[b]Number of mice per group.
[c]$P < 0.05$, significant differences between CO and MO groups.

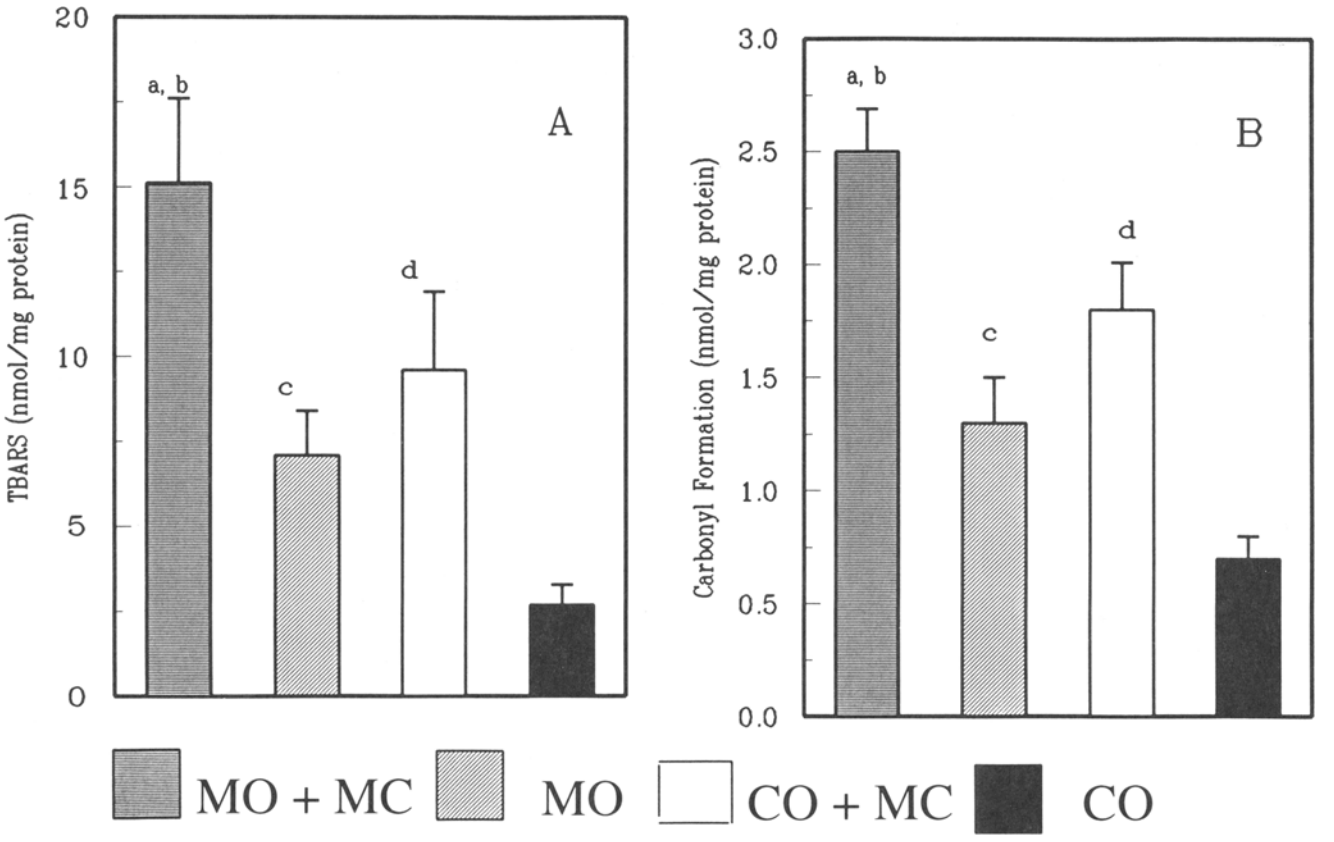


FIG. 3. Effects of feeding a diet high in MO, or low in CO and MC treatment on tumor (A) lipid peroxidation and (B) protein oxidation. Statistically significant differences were (A) in lipid peroxidation between MO + MC and MO groups (a: $P < 0.01$), between MO + MC and CO + MC groups (b: $P < 0.01$), between MO and CO groups (c: $P < 0.01$), and between CO + MC and CO groups (d: $P < 0.01$) and in (B) protein oxidation between MO + MC and MO groups (a: $P < 0.001$), between MO + MC and CO + MC groups (b: $P < 0.01$), between MO and CO groups (c: $P < 0.01$), and between CO + MC and CO groups (d: $P < 0.01$). Abbreviations as in Figure 1.

significantly decreased in the MO-fed group, indicating that high levels of dietary menhaden oil resulted in predisposing the tumors to oxidative stress. Although the mechanism by which oxidative stress inhibits tumor growth is not clear, lipid peroxidation products are reported to be capable of decreasing cell proliferation by damaging cell membranes, changing cellular structures, and inactivating cellular macromolecules such as key enzymes and other protein factors (41–44). However, under extreme oxidative conditions, lipid peroxidation may lead to cell death (45–48). Therefore, the observed inhibition of tumor growth by MO in this study may in part be attributed to the increased susceptibility of tumor cells to lipid peroxidation by altering the level of unsaturation of their membranes. This conclusion is consistent with the findings of Gonzalez *et al.* (5) who related the inhibition of tumor growth in animals fed fish oil to lipid peroxidation by preventing the fish oil inhibition of tumor growth by feeding high levels of α-tocopherol.

We previously reported that feeding a 25% CO diet also increased tumor oxidative stress as measured by increased tumor antioxidant enzyme activities and lipid peroxidation. However, contrary to findings reported here, in comparison to the 5% CO diet, the 25% CO diet enhanced human mammary carcinoma growth (14). The reason for this difference in tumor growth between the 25% CO (14) and 25% MO (Table 4) dietary regimes may be the difference in lipid peroxidation found in the tumors fed the different high-fat diets. Under certain conditions, metabolites of PUFA peroxidation actually activate cellular proliferation (49), whereas the cytotoxic effects of lipid peroxidation occur only under extreme oxidative conditions (45–48). The data in Table 4 indicate that lipid peroxidation in the tumors from animals fed 25% CO increased 25% over the 5% CO controls, whereas in the 5% CO + 20% MO groups, lipid peroxidation was increased 300%. Therefore, we speculate that the differences between stimulating tumor growth in the 25% CO diet and inhibiting tumor growth in the 5% CO + 20% MO diet is the extent of lipid peroxidation and production of toxic peroxidation byproducts. As reviewed by Esterbauer *et al.* (50), ω6 PUFAs, such as linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$), yield 4-hydroxynonenal (HNE), 4-hydroperoxynonenal, and 4,5-dihydroxydecenal, whereas the ω3 PUFAs, like docosahexaenoic acid ($C_{22:6}$), yield 4-hydroxyhexenal, 4-hydroperoxy hexenal, and 4,5-dihydroxy heptenal. The 4-hydroxy alkenals have widespread activity, including formation of cellular membrane bleb leading to cell death in Ehrlich ascites,

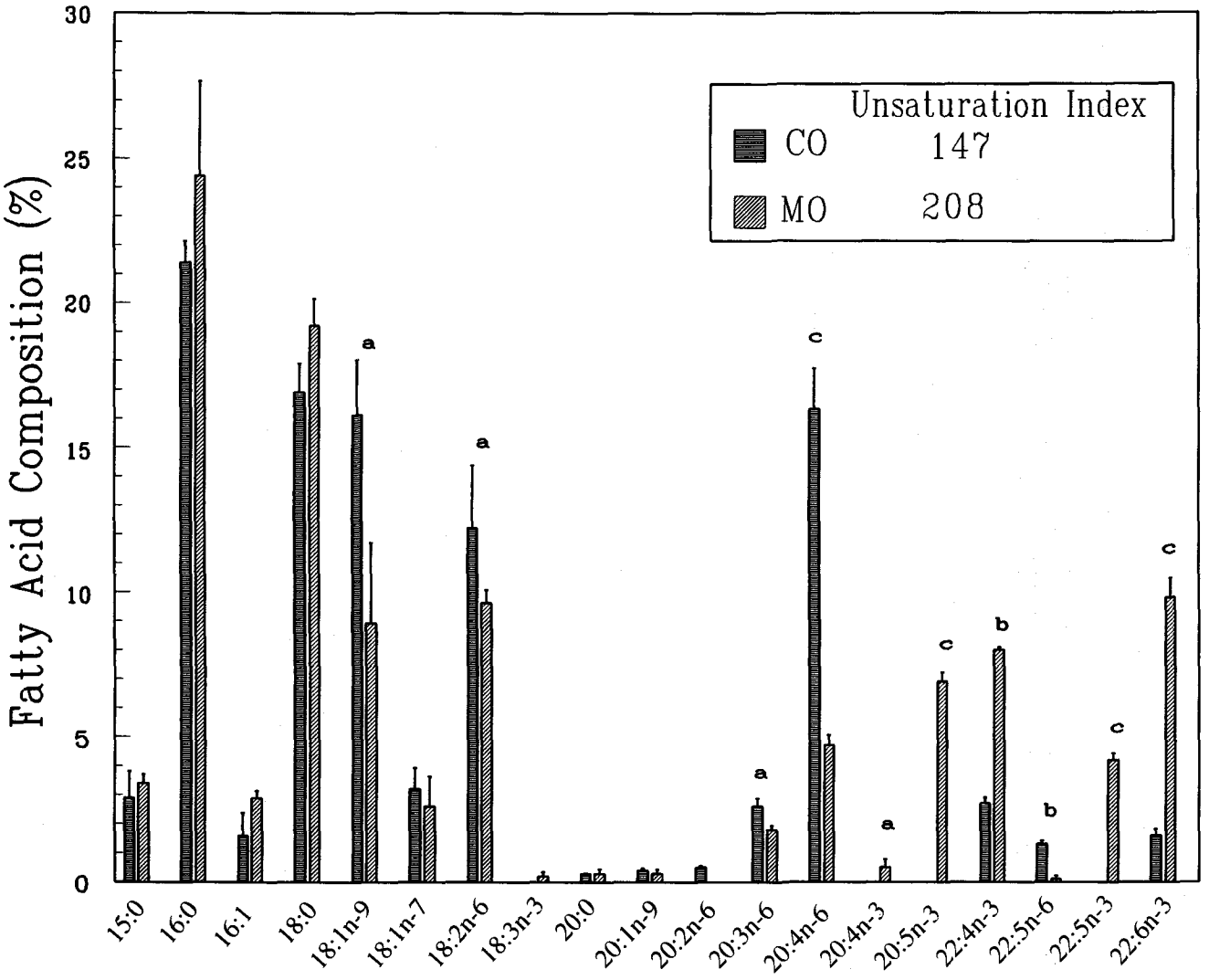


FIG. 4. Effects of feeding a diet high in MO and low in CO on tumor fatty acid compositions. Statistical differences between MO and CO groups were: a, $P < 0.05$; b, $P < 0.01$; and c, $P < 0.001$. The unsaturation index is calculated by multiplying the fatty acid percentage with the number of the double bond it has. Abbreviations as in Figure 1.

inhibition of glycolysis, respiration, depletion of GSH and protein thiols, protein syntheses, and DNA synthesis (51–56). Thus, the production of these toxic lipid peroxidation byproduct could account for the depressed tumor growth observed by MO feeding.

Based on the different fatty acid distribution in the tumor from the CO- and MO-fed groups (Fig. 4), we would expect different lipid peroxidation products to be formed in the tumors from each group. The extent to which these different products are formed in each tumor has not yet been determined, and therefore their contribution to the different growth patterns of tumor from the two groups is at present unknown. On the other hand, CO contains high amounts of n-6 PUFAs, which can enhance the synthesis of prostaglandins after incorporation into the cell membranes; and prostaglandins have been proved to stimulate tumor proliferation (1–4). In fact, the enhanced tumor growth by high–n-6 PUFA diets could be partially reversed by the treatment of indomethacin, a prostaglandin synthase inhibitor. However, n-3 PUFAs (such as MO) have been shown to inhibit prostaglandin synthesis by either directly inhibiting prostaglandin synthase or competing for the substrate binding site with arachidonic acid (which is the natural substrate for prostaglandin synthase to produce prostaglandins) (1–4). In this study, MO feeding resulted in a threefold decrease of arachidonic acid levels in the tumor cell membrane compared to the 5% CO (Fig. 4). The decreased arachidonic acid and increased incorporation of n-3 PUFAs in the tumor membranes of the MO-fed animals may affect the production of prostaglandins in the tumor cells.

The most interesting finding in this study is the relationship between dietary fat and tumor chemotherapy. In our earlier study (14), a high-CO diet (25% CO) significantly increased the tumor response to MC chemotherapy. We believed that this was due to activation of the MC metabolizing system by the high-fat diet, because some well-known MC bioreductive-activating enzyme activities such as DTD, CB5R, and

TABLE 4
Comparison Between the Effects of High–Corn Oil Diet (25%) and High–Menhaden Oil Diet[a]

	HCO	HCO + MC	MO	MO + MC
Tumor weight (mg) at day 30	4100 ± 1950	200 ± 69	2016 ± 875	15 ± 17
T/C ratio[b]		5		1
Combined T/C ratio[c]		5	0.37	
% of TBARS increase[d]	25	160	300	600
DT-diaphorase activity (nmol/min/mg)	16.2 ± 2.0	19.2 ± 2.9	30.9 ± 3.6	38.0 ± 4.3
Xanthine oxidase activity (pmol/min/mg)	130 ± 13	190 ± 14	143 ± 23	289 ± 52

[a]Data were collected from previous study (Ref. 14) and present study. HCO was the high–corn oil diet containing 25% corn oil in the diet; MO was the menhaden oil diet containing 20% menhaden oil + 5% corn oil.
[b]The percentage treatment/control ratio (T/C ratio) was calculated by the mean tumor weight of the MC-treated group divided by the mean tumor weight of the respective control group (MC untreated) multiplied by 100.
[c]The combined T/C ratio was calculated by the mean tumor weight of the MC-treated groups divided by the mean tumor weight of the HCO control group multiplied by 100.
[d]The data were obtained by the mean thiobarbituric acid reactive substances (TBARS) value in the respective dietary groups (HCO or MO) divided by the mean TBARS value from the low-fat (5% corn oil) control group mutiplied by 100.

XO/XD activities were enhanced by the high–CO diet. In the present study, human mammary carcinoma MX-1 was more sensitive to MC in the MO-fed group than in the control group (CO) (Table 2). MC treatment caused a significant depression in tumor growth compared to CO (Table 2) or 25% CO group (14) (Table 4). Tumor DTD and XO were also activated by dietary MO, but CB5R and XD activities were not increased (Fig. 1). These results indicate that feeding a high amount of dietary fat could alter the tumor susceptibility to MC treatment by enhancing certain drug activating enzyme systems directly in the tumor. A diet containing different fatty acids may have a different effect on various tumor enzymes. For example, we previously reported that feeding a high–CO diet (25% CO, w/w) increased the activities of CB5R and XD in addition to DTD and XO in human mammary carcinoma MX-1 (14); whereas in this study, the activities of CB5R and XD were not increased by high dietary MO. Since MO is associated with increased responsiveness to MC therapy, these findings tend to rule out the role of intratumor CB5R and XD in the MO-induced increased response to MC, although both enzyme activities were increased by treatment with MC. In this regard, the highest increase in MC-activating enzymes was XD, where treatment resulted in a threefold increase in tumor XD activity in MO-fed mice.

DTD catalyzes a two-electron reduction of MC resulting in the formation of the corresponding hydroquinone structure (10). Although it has been controversial, the role of DTD in the bioreductive activation of MC (57,58) has been clarified by using purified enzymes in cell-free systems. Metabolism of MC to DNA-reactive species by DTD is pH dependent in that it has been reported that the DNA cross-linking induced by MC increases as the intracellular pH is lowered. In addition, the bioreductive-activation of MC by DTD seems more efficient under aerobic conditions than under hypoxic conditions (for review, see Ref. 59). Both high levels of dietary CO (14) and MO increased endogeneous DTD activity in human mammary carcinoma MX-1, while increasing sensitivity to MC. Both diets support increased DTD activities following MC treatment, which support a major role for DTD in the bioreductive activation of MC in this model. XO activity was increased in tumors from the MO-fed group, and MC treatment further increased XO activity. These findings indicate that XO also plays a role in MC activation. We cannot rule out XD as a possible activation enzyme of MC, in that it was always activated following MC treatment on either the 25% CO diet (14) or the MO diet (Fig. 1). Under aerobic conditions, hydroquinone can be reoxidized to the semiquinone free radical or quinone structure with subsequent formation of superoxide, hydrogen peroxide, and hydroxy radical (7). This results in increased oxidative stress in the tumor which could contribute to the antineoplastic activity of MC. In fact, Pritsos *et al.* (60) reported that the toxicity of MC toward EMT-6 cells was decreased in a dose-dependent manner with increasing activity of SOD, a well-known free radical scavenge enzyme. In the present study, MC treatment caused significant levels of oxidative stress in the tumor from both MO and CO groups, as indicated by the increased antioxidant enzyme activities (SOD, CAT, GPOx, and GSTPx) and lipid peroxidation and protein oxidation. In addition, tumor GR was inhibited by MC treatment (Fig. 2D), which would be expected to increase tumor oxidative stress. This finding is consistent with the findings of others (61) that quinones can inhibit the activities of thiol containing enzymes. The role of inhibition of GR in the antitumor activity of MC requires further investigation, but is consistent with the decreased GSH levels in the tumors (Table 3). Collectively, these results indicate clearly that MC treatment caused oxidative stress in the tumor. As expected, the tumors from the MO-fed group were more susceptible to MC-induced oxidative stress because their membrane PLs were more unsaturated.

In summary, high intake of fats rich in long-chain n-3 PUFAs such as MO will increase the level of unsaturation in tumor membrane PLs. This results in tumor lipids possessing a high degree of unsaturation, which makes them more susceptible to endogenous and exogenous lipid peroxidation. When exogenous oxidative stress is induced by prooxidant drugs such as MC treatment, increased lipid peroxidation, protein oxidation, and increased responsiveness to MC

chemotherapy is observed. In addition, increased tumor activities of DTD and XO, two of the proposed MC bioreductive activating enzymes, resulted from feeding a high-MO diet, indicating that nutritional intervention involving this type of dietary fat may increase the therapeutic response to prooxidant therapy.

REFERENCES

1. Welsch, C.W. (1992) Relationship Between Dietary Fat and Experimental Mammary Tumorigenesis: A Review and Critique, *Cancer Res. 52*, 2040s–2048s.
2. Carroll, K.K. (1992) Dietary Fat and Breast Cancer, *Lipids 27*, 793–797.
3. Welsch, C.W. (1987) Enhancement of Mammary Tumorigenesis by Dietary Fat: Review of Potential Mechanisms, *Am. J. Clin. Nutr. 45*, 192–202.
4. Pariza, M.W. (1988) Dietary Fat and Cancer Risk: Evidence and Research Needs, *Ann. Rev. Nutr. 8*,167–183.
5. Gonzalez, M.J., Schemmel, R.A., Dugan, L., Jr., Gray, J.I., and Welsch, C.W. (1993) Dietary Fish Oil Inhibits Human Breast Carcinoma Growth: A Function of Increased Lipid Peroxidation, *Lipids 28*, 827–832.
6. Kaasgaard, S.G., Holmer, G., Hoy, C-E, Behrens, W.A., and Beare-Rogers, J.L. (1992) Effects of Dietary Linseed Oil Marine Oil on Lipid Peroxidation in Monkey Liver *in vivo* and *in vitro*, *Lipids 27*, 740–745.
7. Powis, G. (1989) Free Radical Formation by Antitumor Quinones, *Free Radical Biology & Medicine 6*, 63–101.
8. Sinha, B.K. (1989) Free Radicals in Antitumor Drug Pharmacology, *Chem. Biol. Interactions 69*, 293–317.
9. Sinha, B.K., and Mimnaugh, E.G. (1990) Free Radicals and Anticancer Drug Resistance by Certain Tumors, *Free Radical Biology & Medicine 8*, 567–581.
10. Riley, R.J., and Workman, P. (1992) DT-Diaphorase and Cancer Chemotherapy, *Biochem. Pharmacol. 43*, 1657–1669.
11. Yang, C.S., Brady, J.F., and Hong, J.Y. (1992) Dietary Effects on Cytochrome P450, Xenobiotic Metabolism, and Toxicity, *FASEB J. 6*, 737–744.
12. Yoo, J-S.H., Hong, J.Y., Ning, S.M., and Yang, C.S. (1990) Roles of Dietary Corn Oil in the Regulation of Cytochrome P450 and Glutathione S-Transferase, *J. Nutr. 120*, 1718–1726.
13. Yoo, J-S.H., Ning, S.M., Pantuck, C.B., Pantuck, E.J., and Yang C.S. (1991) Regulation of Hepatic Microsomal Cytochrome P450IIE1 Level by Dietary Lipids and Carbohydrates in Rats, *J. Nutr. 121*, 959–965.
14. Shao, Y., Pardini, L., and Pardini, R.S. (1994) Enhancement of the Antineoplastic Effect of Mitomycin C by Dietary Fat, *Cancer Res. 54*, 6452–6457.
15. American Institute of Nutrition (1977) Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies, *J. Nutr. 107*, 1340–1348.
16. American Institute of Nutrition (1980) Second Report of the Ad Hoc Committee on Standards for Nutritional Studies, *J. Nutr. 110*, 1726.
17. Birt, D.F., White, L.T., Choi, B., and Pelling, J.C. (1989) Dietary Fat Effects on the Initiation and Promotion of Two-Stage Skin Tumorigenesis in the SENCAR Mouse, *Cancer Res. 49*, 4170–4174.
18. Borgeson, C.E., Pardini, L., Pardini, R.S., and Reitz, R.C. (1989) Effects of Dietary Fish Oil on Human Mammary Carcinoma and on Lipid-Metabolizing Enzymes, *Lipids 24*, 290–295.
19. Ernster, L. (1967) DT-Diaphorase, *Methods in Enzymology 10*, 309–317.
20. Benson, A.M., Hunkler, M.J., and Talalay, P. (1980) Increase of NAD(P)H:Quinone Reductase by Dietary Antioxidants: Possible Role in Protection Against Carcinogenesis and Toxicity, *Proc. Natl. Acad. Sci. USA. 77*, 5216–5220.
21. Sukiman, S.A., and Stevens, J.B. (1976) Purification of Xanthine Dehydrogenase: A Rapid Procedure with High Enzyme Yield, *Arch. Biochem. Biophys. 258*, 2040s–2048s.
22. Masters, B.S.S., Williams, C.H., and Kamin, H. (1967) The Preparation and Properties of Microsomal TPNH-Cytochrome C Reductase from Pig Liver, *Methods in Enzymology 10*, 565–573.
23. Yasukochi, Y., and Masters, B.S.S. (1976) Some Properties of a Detergent Solubilized NADPH-Cytochrome C (Cytochrome P450) Reductase Purified by Biospecific Affinity Chromatography, *J. Biol. Chem. 251*, 5337–5344.
24. Oberely, L.W., and Spitz, D.R. (1984) Assay of Superoxide Dismutase in Tumor Tissue, *Methods in Enzymology 105*, 457–464.
25. Aebi, H. (1984) Catalase *in vitro*, *Methods in Enzymology 105*, 121.
26. Strauss, R.G., Snyder, E.L., Wallace, P.D., and Rosenburg, T.G. (1980) Oxygen Detoxification Enzymes in Neutrophils of Infants and Their Mothers, *J. Lab. Clin. Med. 95*, 897.
27. Prohaska, J.R., Oh, S-H., Hockstra, W.G., and Ganther, H.E. (1977) Glutathione Peroxidase: Inhibition by Cyanide and Release of Selenium, *Biochem. Biophys. Res. Comm. 74*, 64–71.
28. Racker, E. (1955) Glutathione Reductase, *Methods in Enzymology 2*, 722.
29. Mannervick, B., and Guthenberg, C. (1981) Glutathione Transferase: Human Placenta, *Methods in Enzymology 77*, 231–235.
30. Buege, J.A., and Aust, S.D. (1978) Microsomal Lipid Peroxidation, *Methods in Enzymology LII*, 302–310.
31. Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A-G., Ahn, B., Shalteil, S., and Stadtman, E.R. (1990) Determination of Carbonyl Content in Oxidatively Modified Proteins, *Methods in Enzymology 77*, 231–235.
32. Fariss, M.W., and Reed, D.J. (1987) High-Performance Liquid Chromatography if Thiols and Disulfides: Dinitrophenol Derivatives, *Methods in Enzymology 143*, 101–109.
33. Aksoy, M., Berger, M. R., and Schmahl, D. (1987) The Influence of Different Levels of Dietary Fat on the Incidence and Growth of MNU-Induced Mammary Carcinoma in Rats, *Nutr. and Cancer 9*, 227–235.
34. Gabor, H. and Abraham, S. (1986) Effect of Dietary Menhaden Oil on Tumor Cell Loss and the Accumulation of Mass of a Transplantable Mammary Adenocarcinoma in BALB/c Mice, *J. Natl. Cancer Inst. 76*, 1223–1229.
35. Hudson, E.A., Beck, S.A., and Tisdale, M.J. (1993) Kinetics of the Inhibition of Tumor Growth in Mice by Eicosapentaenoic Acid Reversed by Linoleic Acid, *Biochem. Pharmac. 45*, 2189–2194.
36. Ip, C., Carter, C.A., and Ip, M.M. (1985) Requirement of Essential Fatty Acid for Mammary Tumorigenesis in the Rat, *Cancer Res. 45*, 1997–2001.
37. Burns, A., Lin, Y-G., Gibson, R., and Jamieson, D. (1991) The Effect of a Fish Oil Enriched Diet on Oxygen Toxicity and Lipid Peroxidation in Mice, *Biochem. Pharmacol. 42*, 1353–1360.
38. Javouhey-Donzel, A., Guenot, L., Naupoil, V., Rochette, L., and Rocquelin, G. (1993) Rat Vitamin E Status and Heart Lipid Peroxidation: Effect of Dietary α-Linolenic Acid and Marine n-3 Fatty Acids, *Lipids 28*, 651–655.
39. Chen, L-C., Boissonneault, G., Hayek, M.G., and Chow, C.K. (1993) Dietary Fat Effects on Hepatic Lipid Peroxidation and Enzymes of H_2O_2 Metabolism and NADPH Generation, *Lipids 28*, 657–662.
40. Cho, S-H., and Choi, Y-S. (1994) Lipid Peroxidation and Antioxidant Status Is Affected by Different Vitamin E Levels When Feeding Fish Oil, *Lipids 29*, 47–52.
41. Gonzalez, M.J. (1992) Lipid Peroxidation and Tumor Growth: An Inverse Relationship, *Med. Hypotheses 38*, 106–110.

42. Spector, A.A., and Yorek, M.A. (1985) Membrane Lipid Composition and Cellular Function, *J. Lipid Res. 26*, 1015–1035.
43. Spector, A.A., and Burns, C.P. (1987) Biological and Therapeutic Potential of Membrane Lipid Modification in Tumors, *Cancer Res. 47*, 4529–4537.
44. Farber, J.L., Kyle, M.E., and Cleman, J.B. (1990) Mechanisms of Cell Injury by Activated Oxygen Species, *Lab. Invest. 62*, 670–679.
45. Roubal, W.T. and Tappel, A.L. (1966) Damage to Proteins, Enzymes, and Amino Acids by Peroxidizing Lipids, *Arch. Biochem. Biophys. 113*, 5–8.
46. Reiss, U., and Tappel, A.L. (1973) Fluorescent Product Formation and Changes in Structure of DNA Reacted with Peroxidizing Arachidonic Acid, *Lipids 8*, 199–202.
47. Dianzani, M.U. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D.C., and Slater, T.F., eds.) pp. 129–158, Academic Press, London.
48. Bull, A.W., Nigro, N.D., and Marnett, J.J. (1988) Structural Requirements for Stimulation of Colonic Cell Proliferation by Oxidized Fatty Acids, *Cancer Res. 48*, 1771–1776.
49. King, M.M., Balley, D.M., Gibson, D.D., Pitha, J.V., and McCay, P.B. (1979) Incidence and Growth of Mammary Tumors Induced by 7,12-Dimethyl-benz[a]anthracene as Related to the Dietary Content of Fat and Antioxidant, *J. Natl. Cancer Inst. 63*, 657–663.
50. Esterbauer, H., Schaur, R.J., and Zollner, H. (1991) Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde, and Related Aldehydes, *Free Rad. Biol. Med. 11*, 81–129.
51. Schauenstein, E. (1982) Effects of Low Concentrations of Aldehydes on Tumor Cells and Tumor Growth, in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D.C., and Slater T.F., eds.) pp. 159–171, Academic Press, London.
52. Khoschsorur, G., Schaur, R., Schauenstein, E., Tillian, H.M., and Reiter, M. (1981) Intra-Cellular Effects of Hydroxy Alkenals on Animal Tumors, *Z. Naturforsch 36*, 572–578.
53. Haupthlorenz, S., Esterbauer, H., Moll, W., Pumpel, R., Schauenstein, E, and Puschendorf, B. (1985) Effects of the Lipid Peroxidation Product 4-Hydroxy Nonenal and Related Aldehydes on Proliferation and Viability of Cultured Ehrlich Ascites Tumor Cells, *Biochem. Pharmac. 34*, 3803–3809.
54. Bickis, I. J., Schauenstein, E., and Taufer, M. (1968) Effects of Hydroxypentenal on the Metabolism of Tumor Cells and Normal Cells, *Monatsa. Chem. 100*, 1077–1104.
55. Cadenas, E., Muller, A., Brigelius, R., Esterbauer, A., and Sies, H. (1983) Effects of 4-Hydroxy Nonenal on Isolated Hepatocytes, *Biochem. J. 214*, 479–487.
56. Haenen, G.R.M.M., Tai Tin Tsoi, J.N.L., Vermeulen, N.P.E., Timmermann, A., and Bast, A. (1987) Hydroxy-2,3-*trans*-nonenal Stimulates Microsomal Lipid Peroxidation by Reducing the Glutathione-Dependent Protection, *Arch. Biochem. Biophys. 259*, 449–459.
57. Workman, P., Walton, M.I., Powis,, G., and Schlager, J.J. (1989) DT-Diaphorase: Questionable Role in Mitomycin C Resistance, But a Target for Novel Bioreductive drugs? *Br. J. Cancer 60*, 800–802.
58. Marshall, R.S., Rauth, A.M., and Paterson, M. (1989) Reply to the Letter from Workman *et al.*, *Br. J. Cancer 60*, 803.
59. Ross, D., Siegel, D., Beall, H., Prakash, A.S., Mulcahy, R.T., and Gibson, N.W. (1993) DT-Diaphorase in Activation and Detoxification of Quinones. Bioreductive Activation of Mitomycin C, *Cancer Metastasis Rev. 12*, 83–101.
60. Pritsos, C.A., Keyes, S.R., and Sartorelli, A.C. (1989) Effect of the Superoxide Dismutase Inhibitor, Diethyldithiocarbamate, on the Cytotoxicity of Mitomycin Antibiotics, *Cancer Biochem. Biophys. 10*, 289–298.
61. Takahashi, N., Schreiber, J., Fischer, V., and Mason, R.P. (1987) Fromation of Glutathione-Conjugated Semiquinones by the Reaction of Quinones with Glutathione: An ESR Study, *Arch. Biochem. Biophys. 252*, 41–48.

[Received May 1, 1995, and in final revised form August 28, 1995; Revision accepted September 5, 1995]

Phospholipid Profiling of Sediments Using Phosphorus-31 Nuclear Magnetic Resonance

Lidia G. Bardygula-Nonn[a], Jerry L. Kaster[a] and Thomas Glonek[b,*]

[a]Center for Great Lakes Studies, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53204
and the [b]MR Laboratory, Midwestern University, Chicago, Illinois 60615

ABSTRACT: A phosphorus-31 nuclear magnetic resonance method has been developed for the determination of aquatic sediment phospholipid profiles that may be generally applied to all soils and deposits containing viable cellular material. A method of scrubbing chloroform/methanol extracts with potassium acid phosphate overcomes adverse signal broadening from the mineral component, permitting eleven sediment phospholipids to be determined at the quantitative level.
Lipids 30, 1047–1051 (1995).

Biomass can account for a significant fraction of sedimentary organic matter (1–3). Evaluation of biomass is coupled to understanding environmental interactions of organisms living in both aquatic sediments and terrestrial soils. The living cells constituting the biomass are the principal processors of both nutritive material and human-generated contaminants. These cells are defined by a cellular membrane composed of phospholipids, the profiles of which are characteristic of each cell (4). A profile of sediment phospholipids is representative of the aggregate living biomass community within a sediment and may closely reflect the ecological status of the community response to perturbation.

The methods used for determining biomass in sediments are numerous; however, none assess the profile of all living organisms present in sediments. A method has been developed that utilizes a phospholipid analysis for determining microbial biomass in sediments (5). The method is based upon the determination of lipid-bound phosphorus that is extracted using a modification of the Folch (6) extraction for total lipid.

The determination of lipid-bound phosphorus is based upon the concept that lysosomal activity released upon cell death rapidly hydrolyzes lipid-bound organic phosphate esters, converting oil-soluble organically-bound phosphate into water-soluble inorganic orthophosphate. Lipid-bound phosphorus, therefore, is a component only of living cells and, as such, a measure of viable biomass.

Lipid-bound phosphorus is almost exclusively contained in the polar head groups of cellular membrane phospholipids. These phospholipids can be profiled using phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy to obtain quantitative separation of the component phospholipids that are characteristic of the tissue from which they have been extracted (4,7,8). It follows that the aggregate cellular component of sediments similarly also could be analyzed to yield phospholipid profiles that are characteristic of a given sediment, reflecting its biological fingerprint.

^{31}P NMR phospholipid spectra obtained from clean fresh tissues are highly resolved and yield narrow ^{31}P signals in the range of 2–3 Hz line width at half-height (4,7,8). Spectra obtained from sediments, however, are broadened to the point of being unusable by the mineral component of sediments, principally iron, that broadens NMR signals through the physical–chemical mechanism of paramagnetic relaxation. We have eliminated this serious line-broadening artifact through extract scrubbing using EDTA in conjunction with potassium acid phosphate. The resulting well resolved ^{31}P NMR spectra can be integrated to produce a quantitative sediment phospholipid profile analysis.

Crude sediment total lipid extracts can be analyzed by ^{31}P NMR. This NMR method provides quantitative phospholipid profiles that may be used to fingerprint sediment types, monitor pollution, classify organisms, or provide insight into the fundamental properties of sediment biochemistry. The basic methodology is simple, requiring only common laboratory chemicals and equipment. The only unusual requirement is access to a high field (4.7 Tesla or greater) NMR spectrometer equipped to detect ^{31}P.

MATERIALS AND METHODS

Collection and extraction protocol. Samples (*ca.* 1 kg) consisting of the top 3 cm of sediment were collected during the fall of 1994 from the Milwaukee Harbor at the Center for Great Lakes Studies (Milwaukee, WI). To isolate the microbial community, sediments were passed through a 500 μm U.S. Standard soil sieve for removal of the macroinvertebrate fauna. Water content was analyzed using freeze-dried 2 g subsamples.

For phospholipid analysis, 40 g samples are stirred with 500–600 mL chloroform/methanol (2:1, vol/vol), using a large magnetic stirrer. After 10 min, stirring is stopped and the liquid phase examined for the presence of a second aque-

*To whom correspondence should be addressed at the MR Laboratory, Midwestern University, 5200 South Ellis Ave., Chicago, IL 60615.

Abbreviations: GPLAS, glycerol plasmalogen; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance.

Copyright © 1995 by AOCS Press

ous phase that may be found floating above the heavier chloroform phase. If a second aqueous phase is present, as may be the case with heavily hydrated samples, additional chloroform/methanol is added with stirring to take up this residual aqueous phase, so that only a single liquid phase is present. Failure to adequately dehydrate the sample will compromise the quantitative aspects of the analysis.

After 24 h of extraction with stirring, the extract is filtered through Schleicher & Schuell # 588 pre-pleated paper filters into a 1-L glass reagent bottle fitted with a screw-top Teflon-sealed cap. A volume of scrubbing reagent (0.2 M K–EDTA plus 0.2 M potassium acid phosphate, KH_2PO_4, pH 6.0) equivalent to 20% of the extract volume is added, the bottle capped, and the mixture stirred for 5 d at a rate sufficient to interdisperse the two liquid phases. After scrubbing, the mixture is placed into a separatory funnel and allowed to set for 24 h to thoroughly clear the bottom (chloroform) phase. The chloroform phase is recovered, evaporated to dryness at 40°C, and taken up in 30 mL chloroform/methanol. If needed, a small amount of water may be added along with the chloroform/methanol to dissolve residual K–EDTA carried over with the chloroform phase. The concentrated extract is placed into a separatory funnel, 6 mL 0.2 M K-EDTA added, the mixture shaken and allowed to separate. The chloroform phase is recovered and evaporated for NMR analysis.

^{31}P NMR phospholipid reagent. A chloroform–methanol–aqueous–Cs–EDTA phospholipid NMR reagent has been formulated for the analysis of phospholipids by ^{31}P NMR spectroscopy (4,7,8,9–11). The analytical medium consists of two reagents: (A) reagent-grade chloroform, containing 5% benzene-d_6 and an appropriate concentration of trimethylphosphate, a chemical-shift and quantification reference; (B) reagent-grade methanol containing 0.2 M aqueous Cs–EDTA, pH 6 (4:1, vol/vol). The final methanol reagent is prepared by dissolving 1 mL of the aqueous EDTA solution in 4 mL methanol.

For ^{31}P NMR analysis, the extract lipid sample is dissolved in 2 mL reagent A and transferred to a 10-mm NMR sample tube; 1 mL reagent B is added, and the combination mixed thoroughly. A small aqueous phase separates and is allowed to rise to the top of the sample (1 min). Ordinarily, there is no need to remove this small aqueous phase, which contains no detectable phospholipids, and its presence above the sample ensures that the proper equilibria are maintained and that the organic solvents will not evaporate significantly during the NMR analysis.

^{31}P NMR spectroscopy. The NMR spectrometer used in this investigation was a heteronuclear GE 500 NB system, operating at 202.4 MHz for ^{31}P. Spectrometer scan conditions used: pulse sequence, one pulse; pulse width, 18 μs (45° spin-flip angle); sweep width, 1,000 Hz; acquisition delay, 500 μs; free-induction decay size, 4,096 channels; interpulse delay, 500 μs; acquisition time, 2.05 s; number of acquisitions, 20,000–80,000. Additionally, a computer-generated exponential filter time-constant introducing 0.6 Hz line broadening was applied to reduce background noise. Each signal in the phospholipid spectral profile was quantified through digital integration and chemical-shift values computed (8). In areas of overlapping signals, such as in the immediate vicinity of 0.0 ppm chemical shift, digital spectral curve resolution techniques were applied that have been described (12). Proton broad-band decoupling also was applied routinely (0.25 watt).

Factors that limit the accuracy of ^{31}P NMR measurements (13–16), ^{31}P NMR spectral profile measurements (17), and NMR measurements of extracted phospholipids (7,8,10,11,18,19) have been discussed. In general, properly calibrated NMR spectra yield accurate quantitative data (9,10,14,18), with errors usually attributable to the formulation of inappropriate standards that deviate in matrix composition from those of the analytical samples in the nature and quantity of solutes, solvents, and for phosphates, counter cations, and pH.

RESULTS

Phosphate scrubbing. The ^{31}P NMR reagent developed for phospholipids, which performs well when applied to a wide variety of tissue extract (4,8) and synthetic samples (7,20,21), failed when it was applied to extracts of aquatic sediments. The top spectrum of Figure 1 shows the result of a ^{31}P NMR analysis of the harbor sediment sample prepared using the same procedures employed for the analysis of animal and plant tissue extracts. These procedures involve chloroform/methanol extraction followed by backwashing against 0.2 M aqueous K–EDTA, subsequent evaporation of the recovered chloroform phase, and analysis in the Cs–EDTA ^{31}P NMR reagent. (Analysis of the simple chloroform/methanol extract appropriately concentrated yields no detectable high-resolution NMR signal, whatsoever.) Only three broad phospholipid signals were detected, and the signal-to-noise ratio was very poor. With phosphate scrubbing, the same sediment specimen yields the bottom spectrum of Figure 1. Signal line widths were markedly narrowed and signal-to-noise greatly improved. Note that the broad features of the top spectrum were preserved in the bottom spectrum. The principal signal of the top spectrum corresponded in the bottom spectrum to the aggregate of the signals from phospholipids diphosphatidylglycerol, phosphatidylethanolamine, and sphingomyelin. The signal at 0.5 ppm corresponded to phosphatidylglycerol, while that at –0.8 ppm corresponded to the combination of alkylacylphosphatidylcholine and phosphatidylcholine. In the top spectrum an indication of the presence of lysophosphatidic acid and phosphatidylinositol also was discerned. Insofar as could be determined through curve resolution and integration (12), the relative areas of the signals in both spectra were equivalent, i.e., phosphate scrubbing did not appear to alter the relative amounts of the phospholipids present; it simply narrowed the respective signal widths. Such a property is consistent with the removal of paramagnetic impurities, principally iron, by phosphate scrubbing.

The assignments (Table 1) of both glycerol plasmalogen (GPLAS) and dimethylphosphatidylethanolamine (4,8) are tentative, principally because of the low amounts of these pres-

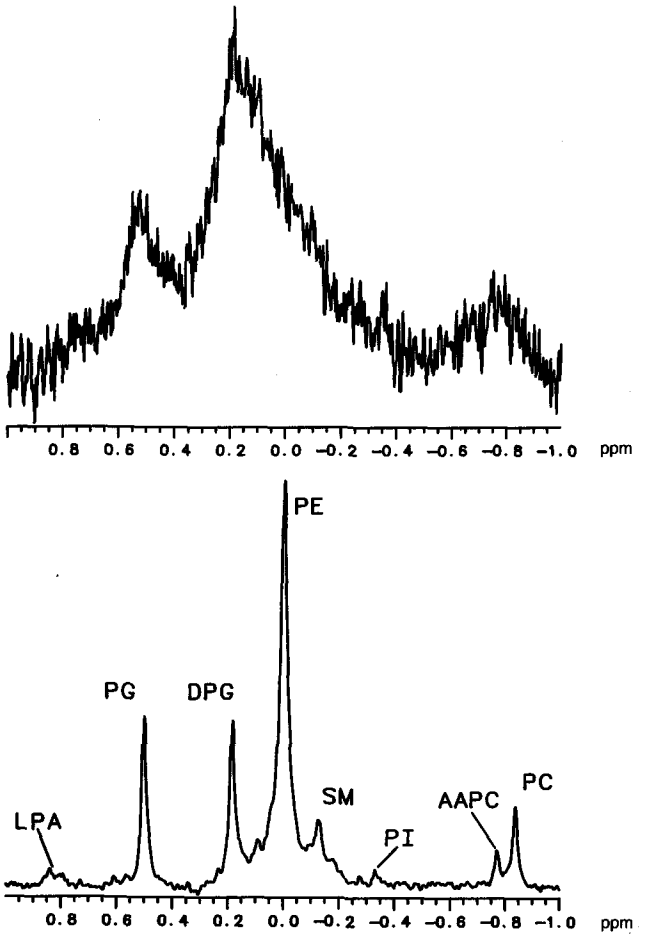


FIG. 1. Phosphorus-31 nuclear magnetic resonance spectral phospholipid profile of Milwaukee Harbor (Milwaukee, WI) sediment. The top spectrum was obtained from a sample that had not been scrubbed by phosphate, the bottom spectrum from a sample that had been scrubbed by phosphate: LPA, lysophosphatidic acid; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; AAPC, alkylacylphosphatidylcholine; PC, phosphatidylcholine.

ent in this sediment. The assignment of GPLAS has an additional uncertainty, since it is not known whether our GPLAS reference samples are true plasmalogens, having the en-ol-ether functional group at the glycerol C_1 position, or the more recently discovered alkyl phospholipids, which have the simple alkyl ether at glycerol C_1.

Precision of the determination. The results from five replicate phospholipid profile determinations of the harbor sediment (Table 1) show a range of variation similar to those obtained with a series of pig lenses (10), which is about five times greater than errors obtained using synthetic laboratory solutions. This larger variability seen with cellular extracts is attributable to variation in the biological sample and can be overcome only through the use of replicate determinations. Standard deviations of the means range from a low of 3.3% for phosphatidylethanolamine to a high of 33.7% for dimethylphosphatidylethanolamine.

Water, oil, and phospholipid content of Milwaukee Harbor sediment specimens. The water content of the sediment samples was 67%. Extractable oil was 0.42% on a wet-weight basis and 1.28% on a dry-weight basis. The phospholipid content was 0.0021% on a wet-weight basis (0.0064% on a dry-weight basis), which corresponded to a phospholipid content in the isolated oil of 0.50%. This amount corresponded to 0.84 mg phospholipid. The number of millimoles phospholipid (assumed molecular weight 789) was 1.07.

DISCUSSION

Sediment specimens. Unlike the analysis of discrete tissue, such as the lens of the eye (10) or cells in tissue culture (22), the analysis of sediments is an analysis of a community. The phospholipid profiles, therefore, are representative of the cellular types that make up the sediment community, and they reflect the weight-fraction of each cell type present. The ^{31}P NMR analysis of sediment profiles biomass at the cellular grade of organization.

Extraction and phosphate scrubbing. After transport of sediment specimens from the field to the MR Laboratory at Midwestern University (Chicago, IL), a sieve is used to remove large debris. The sieving procedure has limitations as to just what is excluded and the degree of sample homogeneity. These limitations can be controlled by using large samples, by thoroughly mixing the sieved specimen, and by repeating the determinations. We have found with the sensitivity inherent in our older spectrometer, that, at the practical level, a 40 g wet-weight sample of sieved sediment is required. This corresponds to approximately 1 cm of sediment core section obtained using a benthos 3-inch diameter corer. Thinner core sample sections could be analyzed with a proportionately increased core diameter.

There must be only one liquid phase present during the initial chloroform/methanol extraction. Extractions carried out overnight with stirring are recommended, since these produce reasonably precise quantitative data (Table 1), although useful extractions can be carried out in about 10 min.

There was a considerable amount of ferromagnetic material in the sediment samples examined, as was evident by the accumulation of such material on the poles of the magnetic stir bars used to mix the samples during extraction. It is clear that iron was the principal element responsible for the NMR line broadening observed in unscrubbed phospholipid samples.

Filtration rates, though slow, were reasonable, even with finely divided samples. The extracts may be heavily pigmented (brown–black, harbor) or only lightly pigmented (light yellow, some river samples). The type or degree of pigmentation does not appear to have any effect on the phospholipid analysis and does not change during the phosphate scrubbing procedure.

Vigorous mixing is required during the phosphate–EDTA scrubbing step. The reaction is carried out using two immiscible liquid phases, with the phosphate being restricted to the

TABLE 1
Replicate Phospholipid Profile Determinations of the Milwaukee Harbor Sediment[a]

Phospholipid	^{31}P NMR chemical shift (ppm)	Phospholipid concentration per replicate determination (mole%)					
		1	2	3	4	5	Means ± SD
LPA	0.81	2.11	2.15	2.45	2.06	2.33	2.22 ± 0.16
GPLAS	0.57	1.12	1.11	1.17	1.03	0.56	1.00 ± 0.25
PG	0.50	11.15	12.28	11.68	12.02	13.17	12.06 ± 0.75
DPG	0.17	13.77	13.83	14.11	14.12	13.51	13.87 ± 0.26
EPLAS	0.09	3.55	3.41	4.03	3.69	3.45	3.63 ± 0.25
PE	0.02	46.33	47.50	46.87	45.16	49.36	47.04 ± 1.55
SM	−0.11	8.66	8.71	9.00	8.71	7.32	8.48 ± 0.66
DIMEPE	−0.18	1.11	0.83	1.06	1.38	0.50	0.98 ± 0.33
PI	−0.33	2.12	1.70	1.56	2.54	1.30	1.84 ± 0.49
AAPC	−0.78	2.82	2.84	2.50	2.69	3.17	2.80 ± 0.25
PC	−0.84	7.33	5.64	5.57	6.60	5.33	6.09 ± 0.84

[a]LPA, lysophosphatidic acid; GPLAS, glycerol plasmalogen; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; EPLAS, ethanolamine plasmalogen; PE, phosphatidylethanolamine; SM, sphingomyelin; DIMEPE, dimethylphosphatidylethanolamine; PI, phosphatidylinositol; AAPC, alkylacylphosphatidylcholine; PC, phosphatidylcholine; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance.

aqueous phase. The EDTA is found in both phases (20), with the bulk concentrated within the aqueous phase. We found that a minimum of 48 h was required for adequate phosphate scrubbing using the harbor sediment. Scrubbing beyond 6 d yielded no further improvement. The reaction probably is driven by the formation of iron phosphate that retains the sequestered iron within the aqueous phase. The EDTA, however, is essential to this process and probably serves as a chemical mediator, since it is capable of moving between the two liquid phases. The crystals that may form during phase separation pose no problem.

Some EDTA is carried within the chloroform phase and appears in the evaporated residue if this is taken to dryness. It can be taken up with the addition of a small amount of water in the transfer procedure for the second washing and is removed essentially completely in the second washing step.

After separation and evaporation to dryness following the second washing step, samples are dissolved in the chloroform–benzene–d_6 NMR reagent A for analysis. At this stage, concentrated samples are so heavily pigmented that they appear black.

Spectral characteristics. Usually the high-resolution phospholipid spectra, such as those shown in Figure 1, are superimposed upon a very broad featureless signal of low amplitude covering a spectral range of about 10 ppm. This signal cannot be reduced by further scrubbing and is characteristic of polymerized and cross-linked (hardened) phospholipids. This broad baseline deformity is easily corrected for accurate spectral integration.

The ^{31}P NMR method for phospholipids depends upon the detection of the phosphorus hetero atom found in the polar head group of each phospholipid. It does not depend upon the nature of the fatty-acid side chains, if these are over six carbon atoms long, or on the presence of double bonds in these side chains, or whether these double bonds are oxidized or otherwise chemically modified (except cross-linked) (8). Because of this property, the ^{31}P method is ideal for the analysis of phospholipids where biological and chemical processes may have altered the double bonds of the incorporated fats, and operations under nitrogen or argon gas are not required.

Sediment phospholipid content. By multiplying the total phospholipid content presented earlier by the mole % (or mole fraction) of each phospholipid in the sample's profile (Table 1), the quantity of each phospholipid in the profile may be calculated. The calculations presented for water, oil, and phospholipid content of Milwaukee Harbor sediment specimens assume that the average fatty-acid side chain is equivalent in molecular-fragment weight to stearoyl, i.e., C_{18} and saturated, and ignores the variations in the molecular-fragment weight of the of the polar head groups. The molecular weight of 789 corresponds to the distearoylphosphatidylcholine.

Phospholipid analysis. From the analytical point of view, the techniques described are designed to be as simple as possible. The idea is to minimize the number of separate chemical manipulations and, thereby, minimize errors. The data are presented as the mole-percent phosphorus present as a given generic species in the total phosphorus detected, which is the simplest and most straightforward manner of presenting the spectroscopic data (17). This method of presenting the data does not require exhaustive phospholipid extraction, only representative extraction, and it corrects internally for quantification errors developed during sample work-up.

The phospholipid profiles obtained contain all members of the broad class of phospholipid biochemicals, the familiar, as well as the new, the bizarre, and the previously unrecognized (4,8). Such an analytical strategy not only saves time, but, more importantly, it provides a total picture of the phospholipid component from which new, useful natural products may be obtained and from which insights into the operant biochemistry may be gleaned.

REFERENCES

1. Alongi, D.M. (1988) Bacterial Productivity and Microbial Biomass in Tropical Mangrove Sediments, *Microb. Ecol. 15*, 59–79.
2. Gillian, F.T., and Hogg, R.W. (1984) A Method for the Estimation of Bacterial Biomass and Community Structure in Mangrove-Associated Sediments, *J. Microbiol. Meth. 2*, 275–293.
3. Moriarty, D.J.W., Boon, P.I., Hansen, J.A., Hunt, W.G., Poiner, I.R., Pollard, P.C., Skyring, G.W., and White, D.C. (1985) Microbial Biomass and Productivity in Seagrass Beds, *Geomicrobiol. J. 4*, 21–51.
4. Glonek, T., and Merchant, T.E. (1995) ^{31}P NMR Phospholipid Profiling, in *Advances in Lipid Methodology* (Christie, W.W., ed.), Vol. 3, pp. 47–94, The Oily Press, Ltd., West Ferry.
5. Findlay, R.H., King, G.M., and Watling, L. (1989) Efficacy of Phospholipid Analysis in Determining Microbial Biomass in Sediments, *Appl. Environ. Microbiol. 55*, 2888–2893.
6. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
7. Meneses, P., and Glonek, T. (1988) High Resolution ^{31}P NMR of Extracted Phospholipids, *J. Lipid Res. 29*, 679–689.
8. Glonek, T. (1994) ^{31}P NMR in the Analysis of Extracted Tissue Phospholipids, in *P-31 NMR Spectral Properties in Compound Characterization and Structural Analysis* (Quin, L.D., and Verkade, J.G., eds.), pp. 283–294, VCH Publishers, New York.
9. Meneses, P., Navarro, J.N., and Glonek, T. (1993) Algal Phospholipids by ^{31}P NMR: Comparing Isopropanol Pretreatment with Simple Chloroform/Methanol Extraction, *Int. J. Biochem. 25*, 903–910.
10. Meneses, P., Para, P., and Glonek, T. (1989) ^{31}P NMR of Tissue Phospholipids: A Comparison of Three Tissue Pre-Treatment Procedures, *J. Lipid Res. 30*, 458–461.
11. Edzes, H.T., Teerlink, T., van der Knapp, M.S., and Valk, J. (1992) Analysis of Phospholipids in Brain Tissue by ^{31}P NMR at Different Compositions of the Solvent System Chloroform–Methanol–Water, *Magn. Reson. Med. 26*, 46–59.
12. Sachedina, S., Greiner, J.V., and Glonek, T. (1991) Membrane Phospholipids of the Ocular Tunica Fibrosa, *Invest. Ophthalmol. Vis. Sci. 32*, 625–632.
13. Van Wazer, J.R., and Glonek, T. (1972) High-Resolution Nuclear Magnetic Resonance, in *Analytical Chemistry of Phosphorus Compounds* (Hallmann, M., ed.), pp. 151–188, John Wiley & Sons, Inc., New York.
14. Burt, T.C., Glonek, T., and Bárány, M. (1976) Analysis of Phosphate Metabolites, the Intracellular pH, and the State of Adenosine Triphosphate in the Intact Muscle by Phosphorus Nuclear Magnetic Resonance, *J. Biol. Chem. 251*, 2584–2591.
15. Bárány, M., and Glonek, T. (1982) Phosphorus-31 Nuclear Magnetic Resonance of Contractile Systems, in *Methods in Enzymology* (Frederiksen, D.L., and Cunningham, L.W., eds.), Vol. 85B, pp. 624–676, Academic Press, Inc., New York.
16. Bárány, M., and Glonek, T. (1984) Identification of Diseased States by P-31 NMR, in *Phosphorus-31 NMR: Principles and Application* (Gorenstein, D., ed.), pp. 511–545, Academic Press, Inc., New York.
17. Klunk, W.E., Xu, C.-J., Panchalingam, K., McClure, R.J., and Pettegrew, J.W. (1994) Analysis of Magnetic Resonance Spectra by Mole Percent: Comparison to Absolute Units, *Neurobiology of Aging 15*, 133–140.
18. Merchant, T.E., and Glonek, T. (1990) ^{31}P NMR of Phospholipid Glycerolphosphodiester Residues, *J. Lipid Res. 31*, 479–486.
19. Pearce, J.M., Shifman, M.A., Pappas, A.A., and Komoroski, R.A. (1991) Analysis of Phospholipids in Human Amniotic Fluid by ^{31}P NMR, *Magn. Reson. Med. 21*, 107–116.
20. Merchant, T.E., and Glonek, T. (1992) ^{31}P NMR of Tissue Phospholipids: Competition for Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} Cations, *Lipids 27*, 551–559.
21. Panchalingam, K., Sachedina, S., Pettegrew, J.W., and Glonek, T. (1991) Al-ATP as an Intracellular Carrier of Al(III) Ion, *Int. J. Biochem. 23*, 1453–1469.
22. Merchant, T.E., Lass, J.H., Roat, M.I., Skelnik, D.L., and Glonek, T. (1990) P-31 NMR Analysis of Phospholipids from Cultured Human Corneal Epithelial, Fibroblast and Endothelial Cells, *Curr. Eye Res. 9*, 1167–1176.

[Received May 30, 1995, and in final revised form September 26, 1995; Revision accepted September 27, 1995]

Urinary Malondialdehyde-Equivalents During Ingestion of Meat Cooked at High or Low Temperatures

Ellen D. Brown[a,*], Virginia C. Morris[a], Donna G. Rhodes[a], Rashmi Sinha[b] and Orville A. Levander[a]

[a]Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA, Beltsville, Maryland 20705 and [b]Environmental Epidemiology Branch, DCE, NCI, NIH, Bethesda, Maryland 20892

ABSTRACT: Excretion of malondialdehyde (MDA)-generating substances in the urine has been suggested as an indicator of *in vivo* lipid peroxidation. However, MDA in the urine also reflects the amount of lipid peroxidation products consumed in the diet. We determined MDA as the thiobarbituric acid (TBA)–MDA complex in urine of 19 healthy adults (10 male and 9 female) fed large quantities (3.6–4.1 g/kg body weight) of ground beef cooked at a low or a high temperature. Subjects ate a controlled diet with no alcohol or nutritional supplements. For 7 d they consumed ground beef cooked at 100°C for 20 min (low-temperature meat) followed by 7 d with meat fried at 250°C for 22 min (high-temperature meat). Prior to the study, subjects consumed their normal free choice diet with moderate amounts of meat. The concentration of MDA in urine at baseline was 2.1 ± 0.3 μmol TBA–MDA equivalents/day (mean ± SEM). After 7 d of low-temperature meat, urinary TBA–MDA equivalents increased to 23.1 ± 1.4 μmol/d. Urinary TBA–MDA equivalents were consistently lower (6.9–8.0 μmol/d) 1, 2, 3, and 7 d after subjects changed to high-temperature meat. After 7 d of treatment, 97% of the MDA-equivalents in the meat was recovered in 24–h urine samples. The low temperature meat had 3–4 times more MDA than did the high-temperature meat. These data indicate that the amount of meat eaten and the cooking procedures used can dramatically alter urinary MDA. Dietary sources of MDA must be controlled if urinary MDA is to be used as an indicator of oxidative stress.
Lipids 30, 1053–1056 (1995).

Malondialdehyde (MDA) is a by-product of the nonenzymatic peroxidation of polyunsaturated lipid, and excretion of substances in urine that can generate MDA has been suggested as a biomarker for *in vivo* lipid peroxidation (1). However, measurement of urinary MDA also includes the contribution from exogenous MDA-generating substances from dietary sources. Foods, especially meats, vary in the amount of MDA-equivalents present (2), and cooking methods can increase the MDA generation in meats (3). Increased urinary excretion of MDA-equivalents has been observed in human subjects ingesting large quantities of cooked meat or fish (4,5).

The extent to which diet influences total urinary MDA must be quantified before urinary MDA can be considered to be a reliable marker for *in vivo* lipid peroxidation. Lipid peroxidation can induce cell damage and contribute to various pathological conditions (6). Furthermore, several studies have suggested that MDA may be mutagenic (7), carcinogenic (8), or form adducts with DNA (9). However, the significance of these data is uncertain (10). The relative importance of consuming MDA-generating substances and any toxicity they might contribute is not clear.

A study designed to clarify the metabolic consequences of ingesting meat cooked at high or low temperatures offered the opportunity to investigate the urinary excretion of MDA-generating substances in adults fed such meats. Subjects were fed known amounts of ground beef cooked by two different methods. This study design allowed us to evaluate how the amount of meat and cooking method influenced urinary MDA under controlled conditions. We also assessed the balance between ingested and excreted MDA-equivalents to determine how quickly and completely dietary MDA was reflected in altered urinary excretion.

MATERIALS AND METHODS

Nineteen nonsmoking adults (10 male and 9 female), aged 27 to 62 yr and weighing 57 to 103 kg participated in a pre-study of self-selected diets and then in a larger controlled diet study concerning cooked meat during January and February of 1993. Volunteers were in good health as determined by a screening history, medical evaluation, and laboratory tests. They were taking no medications or vitamin/mineral supplements. Procedures for this study were approved by the Human Studies Review Committee of the United States Department of Agriculture (Beltsville, MD) and the Institutional Review Boards of Georgetown University School of Medicine (Washington, D.C.) and the National Cancer Institute (Bethesda, MD).

For 14 d preceding the study (self-selected period), subjects were instructed to eat their normal diets. Seven days before the controlled diet period, subjects collected a 24-h urine sample (baseline).

*To whom correspondence should be addressed at the Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA, Room 201, Bldg. 307, BARC-East, Beltsville, MD 20705-2350.

Abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid.

Copyright © 1995 by AOCS Press

During the controlled diet period, subjects consumed breakfast and dinner from Monday through Friday at the Human Studies Facility of the Beltsville Human Nutrition Research Center (Beltsville, MD) under the supervision of a dietitian. Lunches and weekend meals were packaged and taken home. No other food was allowed. Diet soda, coffee or tea, and other noncaloric beverages were permitted. Weighed portions of the foods were adjusted to maintain body weight throughout the study. Subjects ate 3.6 to 4.1 g meat/kg body weight, all of which was eaten at the evening meal and served in combination with sauces during 7 d of rotating diets. At various times throughout the study, subjects collected 12- and 24-h urine samples.

During the first 7-d controlled diet period, all subjects consumed lean ground beef cooked at a low temperature. This was followed by a second 7-d period with meat cooked at a high temperature. The amount of high temperature cooked meat fed to each subject was decreased to compensate partially for its lower moisture content compared to the low-temperature cooked meat.

Freshly ground lean beef (15% fat) was purchased in large batches from the same local grocery store over several weeks. Meat was cooked within 30 h of purchase. The low-temperature meat was prepared in three batches by gently stirring ground beef in a pan heated to 100°C for 20 min until the meat was slightly browned. Then the meat was drained and held in a 90°C conventional oven for 20 min to reduce the moisture content, resulting in a total cooking weight loss of 35%.

The high-temperature meat was prepared from six batches of patties, 110–125 g, approximately 9 cm in diameter, and 1.5 cm thick. Patties were fried on a stainless-steel griddle for 11 min per side for a total of 22 min at 250°C with a total cooking weight loss of 45%. The cooked patties were broken up in a commercial food processor (Robot Coupe USA, Inc., Jackson, MS).

Samples of the two meat preparations were analyzed for moisture by drying to a constant dry weight at 60°C.

After cooking in batches, all meat for one cooking method was combined into a homogeneous mixture. Meat was aliquoted into bags for separate feeding days and immediately frozen at −15°C. For each day of the controlled diet, meat was defrosted overnight in a refrigerator and weighed out for individual subjects who microwaved their portion one to two minutes just before eating.

A single 12-h overnight urine collection was obtained the first 12 h (dinner to following breakfast) after beginning the low-temperature meat treatment. Two 12-h collections per day were obtained on days 1, 2, and 3 after beginning the high-temperature meat treatment; 24-h collections were obtained on day 7 for both treatments. The first 12-h urine collection period (overnight) began at the evening meal and continued until the first void of the following morning. The second 12-h collection period (day) was from breakfast until the evening meal. The 24-h samples covered a breakfast-to-breakfast period and thus represented urine from the second 12 h of one dose and the first 12 h of the next identical dose. Urine was collected in plastic containers and held on ice until delivery at the end of the collection period. Urine volume was estimated by weight (1 g = 1 mL urine), and then aliquots for analysis were immediately frozen at −20°C.

MDA was measured as thiobarbituric acid (TBA)–MDA equivalents, determined by the method of Halliwell and Chirico (11). This method separates the TBA–MDA complex by high-performance liquid chromatography with fluorescence detection. TBA–MDA equivalents are calculated from an acid hydrolyzed standard of 1,1,3,3-tetramethoxypropane that is carried through the same procedure. Ground beef samples were extracted for analysis by gently mixing with 10% trichloroacetic acid to prevent the release of catalytic iron (12).

Significance of differences among experimental time periods was analyzed by one-way analysis of variance and Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

RESULTS

The concentration of MDA-generating substances in low-temperature cooked meat was three times higher than the value in high-temperature cooked meat (Table 1). When these concentrations were adjusted for moisture differences, the concentration of MDA in low-temperature meat was four times higher than that in meat cooked at a high temperature.

Table 2 presents the urinary excretion and percent recovery of measured MDA at various times throughout the study. After 7 d of dietary treatment, 97% as much MDA-generating substance was recovered in the 24-h urine samples as had been ingested that day. Fifty-five percent as much MDA as in the low-temperature meat was recovered in the urine during the first 12 h after ingestion, while 86–90% was recovered for the high-temperature cooked meat.

DISCUSSION

Our results demonstrate that cooking procedures can dramatically change the MDA content of meat, and this is reflected in the urinary excretion of MDA after consumption of such meat. Newburg and Concon (3) showed an approximately 30% increase in levels of MDA for ground beef cooked at a low temperature compared to meats cooked at a high temperature to the same degree of doneness. We demonstrated a 300–400% difference in the MDA content of meat for our cooking protocols comprising two extreme degrees of doneness. The final level of MDA was presumably a function of its formation and loss by volatilization (3). The gentle prolonged cooking used for the low-temperature meat apparently favored a large amount of MDA formation with minimal losses, whereas the extremely high temperature may have increased losses due to volatility or favored the formation of non-TBA reactive material through increased reactivity of MDA with the protein. These data suggest that cooked meats

TABLE 1
Percentage Dry Weight and Concentration of TBA–MDA Equivalents in Cooked Ground Beef Samples[a]

Cooking method	% Dry weight (g dry wt × 100) / (g cooked wt)	TBA–MDA equivalents (μg/g)	TBA–MDA equivalents (nmol/g)
Low temperature[b]	40.4, 40.5	13.4, 14.9	81.7, 90.8
High temperature[c]	56.7, 57.6	4.4, 5.0	27.0, 30.8

[a]All samples were analyzed in duplicate, and concentrations are reported on a cooked weight basis. TBA–MDA, thiobarbituric acid–malondialdehyde.
[b]Cooked at 100°C for 20 min, then held in 90°C oven for 20 min.
[c]Fried as patties at 250°C for 22 min.

warmed for long periods, such as during a buffet or in a cafeteria, may accumulate high concentrations of MDA-generating substances.

Several studies have demonstrated that MDA is a mutagen (7) and possible carcinogen (8). However, only a negligible fraction of the MDA in foods is absorbed in its free form. Most MDA in tissues such as meat is bound to protein as N-ε-(2-propenal) lysine (13). This compound is not mutagenic in the *Salmonella typhimurium* (Ames) test and does not appear to be a source of free MDA in tissues (10). Our analysis measures total MDA and could not distinguish between the free and bound forms. We analyzed the meat samples fed to our subjects for mutagenicity with the Ames test (14) (Sinha, R., and Knize, M., unpublished data) and did not detect revertants during testing of the low-temperature meat. On the other hand, the high-temperature meat produced 1020 revertants/g which may be due to heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, or other pyrolysis products produced during prolonged cooking at a high temperature (15).

Our data indicate that almost as much MDA is excreted in 24 h as is consumed from the cooked meat, and 55–90% of that ingested is voided within the first 12 h. Jacobson *et al.* (4) observed a 50–60% recovery of MDA equivalents after 24 h in five subjects. Although these results suggest that most ingested MDA is readily excreted, more definitive studies (e.g., feeding MDA labeled with ^{14}C) are needed to verify that the amount excreted is a certain proportion of that ingested.

The urinary excretion of 2.1 μmol TBA–MDA equivalents/day observed during the free-living portion of our study is comparable to 1.45 μmol MDA equivalents/day for a 70 kg man calculated from the data of Jacobson *et al.* (4). During the free-living portion of our study, subjects consumed a mean of 131 g of cooked meat per day (data not shown). Assuming a meat consumption of 131 g/d and using the analytical data of Jacobson *et al.* (4) (2–4 μg MDA/g cooked chicken, pork, or beef), we estimate these subjects ingested 1.6–3.2 umol TBA–MDA equivalents/day, considerably less than that given during the controlled feeding period. This intake value corresponds well with our reported mean urinary excretion of 2.1 umol TBA–MDA equivalents/day. Thus, the feeding of a large quantity of meat provided high amounts of exogenous MDA-generating substances which apparently result in excretion of large quantities of TBA-reactive substances co-eluting with TBA–MDA. This variation in background MDA-generating substances in the diet would make assessment of endogenously produced MDA difficult without controlling for this factor.

TABLE 2
Urinary Excretion and Percent Recovery of TBA–MDA Equivalents Consumed in a Diet[a]

Diet	Collection period: 1st 12 h (overnight)	2nd 12 h (day)	24 h Total
		(μmol TBA–MDA equivalents)	
Self-selected	—	—	2.1 ± 0.3^{x}
Low-temperature meat[b]			
Day 1	13.0 ± 0.5^{x} (55 ± 2)[d]	—	—
7	—	—	23.1 ± 1.4^{z} (97 ± 4)
High-temperature meat[c]			
Day 1	6.4 ± 0.3^{y} (90 ± 5)	1.5 ± 0.1^{x} (21 ± 2)	$7.9 \pm 0.4^{y,e}$ (111 ± 6)
2	6.1 ± 0.3^{y} (86 ± 3)	1.7 ± 0.2^{x} (24 ± 3)	$7.8 \pm 0.4^{y,e}$ (109 ± 5)
3	6.2 ± 0.3^{y} (87 ± 4)	1.8 ± 0.2^{x} (25 ± 2)	$8.0 \pm 0.4^{y,e}$ (113 ± 6)
7	—	—	6.9 ± 0.3^{y} (97 ± 3)

[a]Values represent mean ± SEM; n = 19; values in same column with different lettered (x,y,z) superscripts differ significantly ($P < 0.05$). Samples were not collected at time points with missing data; see Table 1 for abbreviations.
[b]23.7 ± 0.7 μmol MDA equivalents consumed/day, analyzed as TBA–MDA equivalents.
[c]7.1 ± 0.2 μmol MDA equivalents consumed/day, analyzed as TBA–MDA equivalents.
[d]Numbers in parentheses represent excreted MDA-generating substances as the percentage of that consumed.
[e]Values were calculated by adding 1st and 2nd 12-h measurements.

Several workers have commented on the possible usefulness of urinary MDA measurement as a method for monitoring oxidative stress *in vivo* (1,16). Any condition thought to increase oxidative stress, such as dietary vitamin E deficiency (17), administration of pharmacological agents such as adriamycin (18) or carbon tetrachloride (19) or induction of diabetes by streptozotocin (20), is known to result in elevated urinary output of MDA-generating substances. Our results show that 24-h of a controlled intake could be sufficient to create a stable baseline of the urinary MDA excretion. If urinary "MDA" is to be used as an indicator of *in vivo* oxidative stress, however, dietary sources of MDA-generating substances must be controlled. Otherwise, dietary sources of MDA could easily mask any changes in urinary MDA-generating substances due to endogenously produced MDA.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Priscilla Steele and the staff of the Human Studies Facility, USDA. This work was supported in part by National Cancer Institute agreement Y01-CP-20521.

REFERENCES

1. Pryor, W.A., and Godber, S.S. (1991) Noninvasive Measures of Oxidative Stress Status in Humans, *Free Radic. Biol. Med. 10*, 177–184.
2. Siu, G.M., and Draper, H.H. (1978) A Survey of the Malonaldehyde Content of Retail Meats and Fish, *J. Food Sci. 43*, 1147–1149.
3. Newburg, D.S., and Concon, J.M. (1980) Malonaldehyde Concentrations in Food Are Affected by Cooking Conditions, *J. Food Sci. 45*, 1681–1687.
4. Jacobson, E.A., Newmark, H.L., Bird, R.P., and Bruce, W.R. (1983) Increased Excretion of Malonaldehyde Equivalents in the Urine After Consumption of Cooked, Stored Meats, *Nutr. Rep. Intl. 28*, 509–517.
5. Nelson, G.J., Morris, V.C., Schmidt, P.C., and Levander, O. (1993) The Urinary Excretion of Thiobarbituric Acid Reactive Substances and Malondialdehyde by Normal Adult Males After Consuming a Diet Containing Salmon, *Lipids 28*, 757–761.
6. Slater, T.F., Cheeseman, K.H., Davies, M.J., Proudfoot, K., and Xin, W. (1987) Free Radical Mechanisms in Relation to Tissue Injury, *Proc. Nutr. Soc. 49*, 1–12.
7. Basu, A.K., and Marnett, L.J. (1983) Unequivocal Demonstration That Malondialdehyde Is a Mutagen, *Carcinogenesis 4*, 331–333.
8. Shamberger, R.J., Andreone, T.L., and Willis, C.E. (1974) Antioxidants and Cancer. IV. Initiating Activity of Malonaldehyde as a Carcinogen, *J. Natl. Cancer Inst. 53*, 1771–1773.
9. Chaudhary, A.K., Nokubo, M., Reddy, G.R., Yeola, S.N., Morrow, J.D., Blair, I.A., and Marnett, L.J. (1994) Detection of Endogenous Malondialdehyde-Deoxyguanosine Adducts in Human Liver, *Science 265*, 1580–1582.
10. Draper, H.H., and Hadley, M. (1990) A Review of Recent Studies on the Metabolism of Exogenous and Endogenous Malondialdehyde, *Xenobiotica 20*, 901–907.
11. Halliwell, B., and Chirico, S. (1993) Lipid Peroxidation: Its Mechanism, Measurement, and Significance, *Am. J. Clin. Nutr. 57*, 715S–725S.
12. Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., and Hadley, M. (1993) A Comparative Evaluation of Thiobarbituric Acid Methods for the Determination of Malondialdehyde in Biological Materials, *Free Radic. Biol. Med. 15*, 353–363.
13. Piche, L.A., Cole, P.D., Hadley, M., van den Bergh, R., and Draper, H.H. (1988) Identification of N-ε-(2-propenal)lysine as the Main Form of Malondialdehyde in Food Digesta, *Carcinogenesis 9*, 473–477.
14. Ames, B.N., McCann, J., and Yamasaki, E. (1975) Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsomal Mutagenicity Test, *Mutat. Res. 31*, 347–364.
15. Furihata, C., and Matsushima, T. (1986) Mutagens and Carcinogens in Foods, *Ann. Rev. Nutr. 6*, 67–94.
16. Dhanakoti, S.N., and Draper, H.H. (1987) Response of Urinary Malondialdehyde to Factors That Stimulate Lipid Peroxidation *in Vivo*, *Lipids 22*, 643–646.
17. Lee, H.S., Shoeman, D.W., and Csallany, A.S. (1992) Urinary Response to *in vivo* Lipid Peroxidation Induced by Vitamin E Deficiency, *Lipids 27*, 124–128.
18. Baird, M.B., Hough, J.L., and Sfeir, G.T. (1993) Accumulation of Malondialdehyde in Mouse Heart Following Acute Dosing with Adriamycin Is Strain-Specific and Unaffected by Cardiac Catalase Status, *Res. Commun. Chem. Pathol. Pharmacol. 80*, 363–366.
19. Bagchi, D., Bagchi, M., Hasoun, E., and Stohs, S.J. (1993) Carbon-Tetrachloride-Induced Urinary Excretion of Formaldehyde, Malondialdehyde, Acetaldehyde and Acetone in Rats, *Pharmacology 47*, 209–216.
20. Gallaher, D.D., Csallany, A.S., Shoeman D.W., and Olson, J.M. (1993) Diabetes Increases Excretion of Urinary Malonaldehyde Conjugates in Rats, *Lipids 28*, 663–666.

[Received December 16, 1995, and in final revised form July 12, 1995; Revision accepted August 28, 1995]

Inhibition of Cholesterol Synthesis and Hepatic 3-Hydroxy-3-Methylglutaryl–CoA Reductase in Rats by Simvastatin and Pravastatin

M. Del Puppo, S. Rauli and M. Galli Kienle*

Department of Medical Chemistry and Biochemistry, University of Milan, 20133 Milan, Italy

ABSTRACT: In this communication we attempt to provide one possible explanation for the observed differences regarding kinetics and distribution between simvastatin and pravastatin. Rats treated with simvastatin or pravastatin exhibited a reduction in the incorporation of [2-^{14}C]acetate into liver cholesterol and displayed lower plasma mevalonate levels as compared to control animals. Moreover, both the total and dephosphorylated 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase (EC 1.1.1.34) activities, particularly 1 h after treatment, were greatly reduced in liver microsomes obtained from simvastatin-treated as compared to control rats. During the same time frame, these parameters were actually elevated with pravastatin treatment. It is known that HMG–CoA reductase synthesis and activity increase following their competitive inhibition. Our results suggest that pravastatin, at 1 h following treatment, was no longer bound to the enzyme; however, it had entered the liver because its inhibitory effect on cholesterol synthesis was manifest at early times after administration. These data provide a plausible rationale for the earlier observation that activity of simvastatin persists longer in plasma than does that of pravastatin.
Lipids 30, 1057–1061 (1995).

Pravastatin and simvastatin are used in the therapy of hypercholesterolemia due to their inhibitory activity on 3-hydroxy-3-methylglutaryl–coenzyme A (HMG–CoA) reductase (EC 1.1.1.34), the key enzyme in cholesterol synthesis. According to previously reported data, the competitive inhibition of HMG–CoA reductase induces a decrease in the rate of cholesterol biosynthesis and is followed by an increase in low density lipoprotein receptors, enhancing the cellular uptake of cholesterol and lowering plasma low density lipoprotein cholesterol (1). An increase of HMG–CoA reductase concentration also follows the competitive inhibition of HMG–CoA reductase activity (2).

Differences between pravastatin and simvastatin in terms of their absorption, distribution, and excretion have been described. Simvastatin is more lipophilic and has been reported to diffuse through membranes, whereas pravastatin is internalized *via* an active transport mechanism (3–5). The different behavior of pravastatin has been ascribed to its hydrophilic nature, which derives mostly from an additional hydroxy group and from the open hydroxyacid chain instead of the lactonized one of simvastatin. The higher specificity for the liver observed for pravastatin relative to other tissues also has been related to these structural features (4). Kinetic data suggest that simvastatin's inhibitory activity may last longer than that of pravastatin because its main 6-hydroxylated metabolite, which is as active as the parent drug, reenters the liver through the enterohepatic circulation (6). In order to obtain direct evidence for the mechanism underlying this long-lasting effect, we have determined the *ex vivo* activity of HMG–CoA reductase in liver microsomes prepared from rats treated with a single dose of either simvastatin or pravastatin. *In vivo* incorporation of labelled acetate and plasma mevalonate levels were used as parameters to check the inhibition of the overall cholesterol synthesis in the treated rats.

The results show that overall cholesterol synthesis was inhibited by both drugs; both drugs reach the liver where they are active in inhibiting cholesterol synthesis. However, binding of simvastatin or its active metabolite to HMG–CoA reductase appears to last longer as compared to pravastatin, which may represent the rationale of the longer half-life of elimination observed for simvastatin as compared to pravastatin (7).

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade. Pravastatin sodium salt (as Selectin) was obtained from Squibb S.p.A. (Rome, Italy), and simvastatin was from Merck Sharp & Dohme Research Lab. (Rahway, NJ). Sodium [2-^{14}C]acetate (56 mCi/mmol) was purchased from Amersham International (Amersham, Bucks, United Kingdom). NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). HMG–CoA and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Scintillation fluids (Ultima Gold) were from Canberra Packard (Meriden, CT).

Animals. Male Sprague-Dawley rats (Charles River, Como, Italy) (180–200 g) were fed a normal rat chow with a

*To whom correspondence should be addressed at Dipartimento di Chimica e Biochimica Medica, Via Saldini, 50, 20133 Milano, Italy.

Abbreviations: HMG–CoA, 3-Hydroxy-3-methylglutaryl–CoA.

Copyright © 1995 by AOCS Press

lighting cycle from 7.00 a.m. to 7.00 p.m. Drugs were given by gavage (20 mg/kg body weight) as solution of 6.5 mg/mL in water for pravastatin (Selectin—Squibb) or as suspension of 20 mg/mL in 0.5% tragacantha gum for simvastatin. Homogeneity of the preparation was tested by measuring pravastatin and simvastatin by gas chromatography/mass spectrometry. Rats were decapitated 1 or 4 h after oral administration (10 a.m.) of pravastatin or simvastatin. Sodium [2-^{14}C]acetate (4.5 μCi; 80 nmoles in 250 μL) was administered orally to the animals, by gavage, 45 min before the sacrifice. Control animals received the vehicle only. Untreated controls also were sacrified for the evaluation of *in vitro* binding experiments.

Blood was collected in tubes containing 4 mM EDTA, and the plasma obtained by centrifugation was stored at −20°C until analyzed for blood mevalonate determination. Livers were quickly removed and weighed. Part of the liver (1–2 g) was immediately frozen at −80°C for sterol radioactivity determination while the remaining was homogenized (1:2.5, wt/vol) in cold 0.3 M sucrose containing 50 mM NaF and 10 mM 2-mercaptoethanol, pH 7.4. The washed microsomal fraction (8) was suspended in 50 mM potassium buffer, pH 7.2, containing 0.3 M KCl, 1 mM EDTA, and 5 mM dithiothreitol. The final suspension contained 3–4 mg microsomal protein/mL determined according to Bradford (9).

HMG–CoA reductase assay. Total activity of the enzyme which is directly related to the amount of enzyme protein was determined as described previously in microsomes pretreated with a phosphoprotein phosphatase. Activity expressed by the enzyme in its dephosphorylated form also was measured in microsomes avoiding treatment with the phosphatase and maintaining the enzyme in the presence of NaF (10,11).

Plasma levels of mevalonate. Plasma mevalonate levels which reflect the overall cholesterol synthesis were determined by gas chromatography/mass spectrometry using selected ion monitoring and deuterated mevalonolactone as internal standard (12).

Cholesterol synthesis from [2-^{14}C]acetate. The liver tissue, stored frozen at −80°C, was homogenized with a glass homogenizer in ice-cold saline (1:2, wt/vol). Pellets obtained after treatment of liver homogenate (4 mL) with $HClO_4$ (480 μL) followed by centrifugation were extracted with chloroform/methanol (2:1, vol/vol) (3 × 4.5 mL) as described previously (13). Aliquots of the extract were counted to measure total lipid radioactivity. The lipid fraction was then saponified with 0.6 N NaOH in 94% ethanol at 60°C for 90 min, and the nonsaponifiable lipids were extracted with hexane. Aqueous phases were acidified and extracted with diethyl ether for the evaluation of incorporation of radioactivity into fatty acids. Residues were dissolved in 1 mL of ethyl acetate, and the incorporation of radioactivity into total and nonsaponifiable lipids and into fatty acids was determined counting 50 μL aliquots of the radioactive samples with a Packard liquid scintillation counter using Ultima Gold (Packard) as the scintillating mixture. Activity was expressed as % of the dose.

In vitro *binding of simvastatin and pravastatin.* Aliquots (5 mL) of liver homogenate (pool of three rat livers) in 0.3 M sucrose with 50 mM NaF and 10 mM 2-mercaptoethanol were added with 0.1 mL of either pravastatin or simvastatin, both as sodium salts, in sucrose buffer in order to obtain for both drugs two concentration levels (6 and 600 nM). Control samples were added with 0.1 mL of sucrose buffer. The suspensions were incubated at 37°C for 10 min. From each sample washed microsomes were then prepared and HMG–CoA reductase activity was measured as described above.

Statistical analysis. Statistical analysis was performed by multiple comparison using the Duncan test. Results are expressed as means ± SD.

RESULTS

The incorporation of sodium [2-^{14}C]acetate into unsaponifiable lipids (Fig. 1) confirms that both simvastatin and pravastatin inhibit the *in vivo* synthesis of cholesterol. Although not statistically significant, there is a suggestion that the inhibition caused by simvastatin was somewhat greater (94 and 81% at 1 and 4 h) than that caused by pravastatin (79 and 72% at 1 and 4 h). The synthesis of fatty acids from labelled acetate was not affected in treated animals relative to controls (data not shown), indicating that the effect of the drugs on cholesterol synthesis was specific.

Mevalonate plasma levels following simvastatin treatment were significantly lower than those of controls both 1 and 4 h after administration of the inhibitor (Fig. 2), whereas following pravastatin there was only a significant difference at 4 h ($P < 0.05$). Mevalonate levels were also significantly lower with simvastatin as compared to those with pravastatin.

Simvastatin lowered both the total and expressed activities of liver HMG–CoA reductase determined *ex vivo* (Fig. 3).

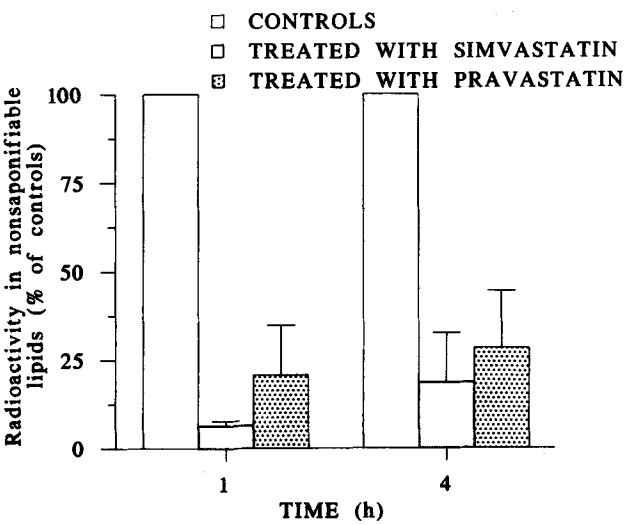


FIG. 1. Inhibition of *in vivo* [2-^{14}C]acetate incorporation into nonsaponifiable liver lipids. Livers of control animals and of rats treated with either simvastatin or pravastatin and [2-^{14}C]acetate were extracted as described in the text. Results are expressed as % of activity found in nonsaponifiable lipids of treated animals assuming 100% that of controls receiving only the vehicle of either simvastatin or pravastatin. Data are means ± SD of values obtained from four rats.

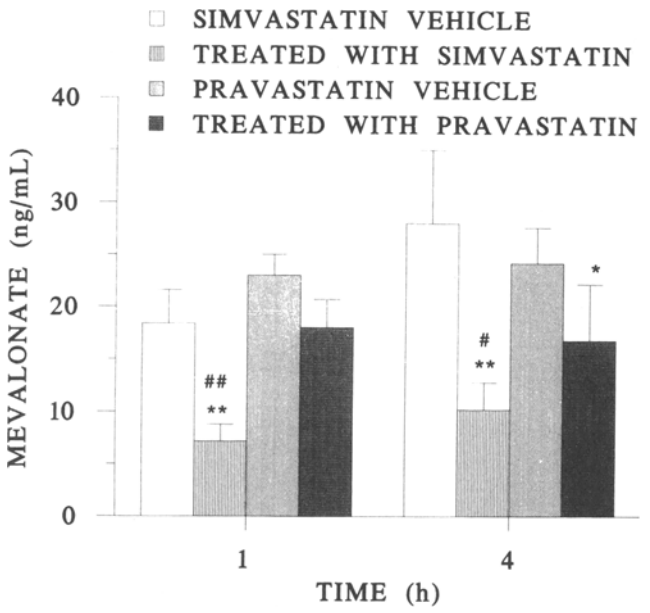


FIG. 2. Mevalonate plasma levels in rats treated with either simvastatin or pravastatin. Data represent mean ± SD of plasma mevalonate concentration (ng/mL) determined in four animals per group. $*P < 0.05$; $**$ $P < 0.01$ vs. control animals; # $P < 0.05$, ## $P < 0.01$ vs. animals treated with pravastatin at the same time.

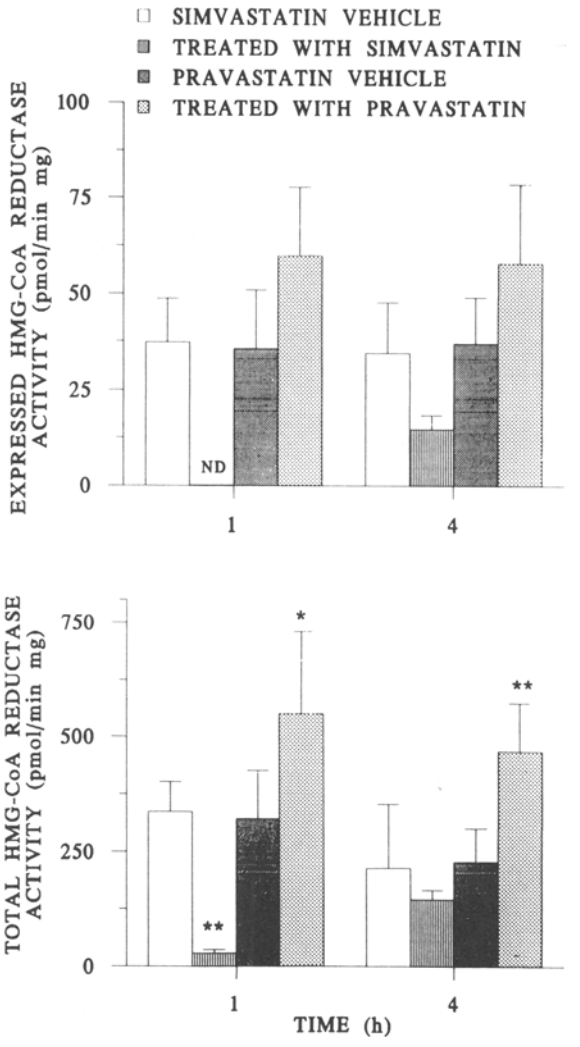


FIG. 3. Hepatic 3-hydroxy-3-methyglutaryl–CoA reductase activity (expressed and total). Activity was measured in the microsomal fractions prepared as described in the text from rats treated with either simvastatin or pravastatin. Results represent mean ± SD of four rats in each group. $*P < 0.05$; $**$ $P < 0.01$ vs. control animals; ND = not detectable.

One hour after treatment, the activity of the expressed dephosphorylated enzyme was below the limits of detection (<4 pmol mevalonic acid min^{-1} mg $protein^{-1}$). At 4 h HMG–CoA reductase activity reached approximately 50% of that of control animals. Nevertheless, due to high variability, statistical significance of the differences was not observed for the expressed form of HMG–CoA reductase. Total activity of the enzyme also was dramatically lowered by simvastatin; more than 90% inhibition of enzyme activity was seen 1 h after the administration of the drug (Fig. 3; $P < 0.01$). At 4 h the activity did not differ significantly from controls. In pravastatin-treated animals a completely different pattern was observed. Total enzyme activity, both 1 h and 4 h after drug administration, was twice that found in controls. There is a suggestion that the expressed form of HMG–CoA reductase also was higher in treated animals both at 1 and 4 h after treatment. Total activity of the enzyme in pravastatin-treated rats 15 and 30 min after treatment was instead 64 and 59% of that of controls, ensuring that lack of inhibition observed at longer times was not attributable to removal of the drug during the preparation of the microsomal fraction.

In order to further ensure that lack of inhibition observed with pravastatin at longer times was not attributable to removal of the drug during the preparation of the microsomal fraction, *in vitro* binding of the two drugs were carried out. Two concentration levels were tested, the higher corresponding to that previously described to occur in liver 2 h after treatment of rats with 25 mg/kg of the two drugs (4), a dose only slightly higher than that used here. Inhibition by pravastatin (Table 1) was lower than by simvastatin, as expected from the higher inhibition constant of the latter drug. Nevertheless, because enzyme inhibition was detectable even with the lower concentration of pravastatin, then pravastatin was not removed during the preparation of microsomes.

DISCUSSION

The marked decrease of labelled acetate incorporation into unsaponifiable lipids by treatment with pravastatin and simvastatin is in good agreement with previously reported data (4). The effect of these drugs on cholesterol biosynthesis was confirmed by mevalonate plasma levels which reflect the total body *in vivo* synthesis and supply an indirect evaluation of modifications of cholesterol synthesis (14,15). Despite the fact that pravastatin effect was significantly lower than that of simvastatin as far as mevalonate plasma levels are concerned, a conclusion about a different therapeutic effect of the two drugs could not be drawn because a single dose was ad-

TABLE 1
Inhibition of 3-Hydroxy-3-Methylglutaryl–CoA Reductase in Microsomes Prepared from Livers Incubated with Pravastatin and Simvastatin[a]

Drug concentration (nM)	Simvastatin		Pravastatin	
	Activity (pmol/min mg protein)	% Inhibition	Activity (pmol/min mg protein)	% Inhibition
0	53 ± 2		53 ± 2	
6	6.6 ± 0.3	88	32 ± 1	40
600	1.6 ± 0.2	97	20 ± 2	62

[a]Liver homogenate of untreated rats were incubated with the two drugs as described in the text. Microsomes isolated after incubation were used to evaluate the expressed enzyme activity as described. Values are expressed as means ± SE.

ministered and evaluation was carried out early after the administration.

In animals and humans it has been shown that pravastatin is actively transported into the liver while its uptake is less efficient in cells where active transport is deficient (5). Moreover it has been reported that pravastatin metabolites are not active as HMG–CoA reductase inhibitors. For the more lipophilic simvastatin, passive transport across membranes occurs, and its main hydroxylated metabolite is as active as the parent drug (6,16). Based on these observations and on the higher affinity of HMG–CoA reductase for simvastatin than for pravastatin, one should expect HMG–CoA reductase in the liver of animals treated with simvastatin to be inhibited for a longer period than in the liver of animals treated with pravastatin. In fact, expressed activity of HMG–CoA reductase in the livers of simvastatin-treated rats was found to be significantly lower than in controls both 1 and 4 h after treatment. With pravastatin, expressed activity was instead slightly higher than in controls. When total activity of the enzyme was measured, which reflects enzyme concentration, differences between simvastatin and pravastatin were even more striking. Both at 1 and 4 h after treatment, activity was dramatically lowered by simvastatin while with pravastatin there was a significant enhancement of the activity.

The increase of total activity of the enzyme observed in pravastatin-treated rats was not unexpected because the inhibition of enzyme activity is known to increase hepatic HMG–CoA reductase messenger RNA levels with the consequent increase of enzyme protein synthesis (2). On the other hand, the increase of the total amount of enzyme was not accompanied by a corresponding increase of overall cholesterol biosynthesis as evidenced by acetate incorporation and mevalonate plasma levels. This might be attributable, in part, to the fact that increase in the level of the enzyme in its active form is not significant.

Simvastatin, however, induced a decrease in the total and expressed activity of the enzyme, thus accounting for the more pronounced decrease observed for the overall synthesis with this drug as compared to pravastatin. Moreover, the inhibition of *ex vivo* HMG–CoA reductase activity strongly suggests that simvastatin is bound to the enzyme even 4 h after administration and therefore is associated with the enzyme in the microsomal fraction used to measure the activity. This is consistent with the inhibition constants reported by Ishida *et al.* (17) which are higher by an order of magnitude for pravastatin ($K_i = 2.3 \cdot 10^{-9}$) than for simvastatin ($K_i = 1.2 \cdot 10^{-10}$) and with the observation that the lipophilic portion of these compounds interacts with a lipophilic portion of the enzyme protein (18). Our explanation for the elevated HMG–CoA reductase activity in pravastatin-treated animals is that the drug is no longer present in the microsomes due to its clearance from the liver prior to harvesting. In fact, inhibition by pravastatin in microsomes prepared from a homogenate incubated in the presence of the drug and inhibition in *ex vivo* experiment at early times after treatment ensured that removal of the drug does not occur from liver after harvesting.

Differences observed here between the two drugs are consistent with the plasma decay curves reported by Morris *et al.* (7) in healthy subjects given a single oral dose of these drugs and represent a direct evidence for the previous hypothesis that the longer half-life of simvastatin is attributable to the higher affinity for HMG–CoA reductase of either the drug or its hydroxylated metabolite.

ACKNOWLEDGMENTS

This work was supported in part by grants of the Italian National Research Council (CNR) and MURST. We are grateful to Andrea Lorenzi for his technical assistance.

REFERENCES

1. Endo, A. (1985) Drugs Inhibiting HMG-CoA Reductase, *Pharmacol. Ther. 31*, 257–267.
2. Ma, P.T., Gil, G., Südhof, T.C., Bilheimer, D.W., Goldstein, J.L., and Brown, M.S. (1986) Mevinolin, an Inhibitor of Cholesterol Synthesis, Induces mRNA for Low Density Lipoprotein Receptor in Livers of Hamsters and Rabbits, *Proc. Natl. Acad. Sci. USA 83*, 8370–8374.
3. Germershausen, J.I., Hunt, V.M., Bostedor, R.G., Bailey, P.J., Karkas, J.D., and Alberts, A.W. (1989) Tissue Selectivity of the Cholesterol-Lowering Agents Lovastatin, Simvastatin and Pravastatin in Rats *In Vivo, Biochem. Biophys. Res. Commun. 158*, 667–675.
4. Koga, T., Fukuda, K., Shimada, Y., Fukami, M., Koike, H., and Tsujita, Y. (1992) Tissue Selectivity of Pravastatin Sodium, Lovastatin and Simvastatin, *Eur. J. Biochem. 209*, 315–319.
5. Komai, T., Shigehara, E., Tokui, T., Koga, T., Ishigami, M., Kuroiwa, C., and Horiuchi, S. (1992) Carrier-Mediated Uptake

of Pravastatin by Rat Hepatocytes in Primary Culture, *Biochem. Pharmacol. 43*, 667–670.
6. Vickers, S., Duncan, C.A., Chen, I.-W., Rosegay, A., and Duggan, D.E. (1990) Metabolic Disposition Studies on Simvastatin, a Cholesterol-Lowering Prodrug, *Drug Metab. Dispos. 18*, 138–145.
7. Morris, M.J., Gilbert, J.D., Hsieh, J.Y.K., Matuszewski, B.K., Ramjit, H.G., and Bayne, W.F. (1993) Determination of the HMG–CoA Reductase Inhibitors Simvastatin, Lovastatin and Pravastatin in Plasma by Gas Chromatography/Chemical Ionization Mass Spectrometry, *Biol. Mass Spectrom. 22*, 1–8.
8. Cighetti, G., Bosisio, E., Galli, G., and Galli Kienle, M. (1983) The Effect of Cholestyramine on Liver HMG–CoA Reductase and Cholesterol 7α-Hydroxylase in Various Laboratory Animals, *Life Sci. 33*, 2483–2488.
9. Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*, 248–254.
10. Cighetti, G., Galli, G., and Galli Kienle, M., (1983) A Simple Model for Studies on the Regulation of Cholesterol Synthesis Using Freshly Isolated Hepatocytes, *Eur. J. Biochem. 133*, 573–578.
11. Galli Kienle, M., Galli, G., Bosisio, E., Cighetti, G., and Paoletti, R. (1984) Evaluation of Enzyme Activities by Gas Chromatography–Mass Spectrometry: HMG–CoA Reductase and Cholesterol 7α-Hydroxylase, *J. Chromatogr. 289*, 267–276.
12. Del Puppo, M., Cighetti, G., Galli Kienle, M., and De Angelis, L. (1989) Measurement of Mevalonate in Human Plasma and Urine by Multiple Selected Ion Monitoring, *Biomed. Environ. Mass Spectrom. 18*, 174–176.
13. Cighetti, G., Del Puppo, M., Paroni, R., Fiorica, E., and Galli Kienle, M. (1987) Pantethine Inhibits Cholesterol and Fatty Acid Syntheses and Stimulates Carbon Dioxide Formation in Isolated Rat Hepatocytes, *J. Lipid Res. 28*, 152–161.
14. Popják, G., Boehm, G., Parker, T.S., Edmond, J., Edwards, P.A., and Fogelman, A.M. (1979) Determination of Mevalonate in Blood Plasma in Man and Rat. Mevalonate "Tolerance" Tests in Man, *J. Lipid Res. 20*, 716–728.
15. Parker, T.S., Mc Namara, D.J., Brown, C., Garrigan, O., and Kolb, R. (1982) Mevalonic Acid in Human Plasma: Relationship of Concentration and Circadian Rhythm to Cholesterol Synthesis Rates in Man, *Proc. Natl. Acad. Sci. USA 79*, 3037–3041.
16. Mauro, V.F. (1993) Clinical Pharmacokinetics and Practical Applications of Simvastatin, *Clin. Pharmacokinet. 24*, 195–202.
17. Ishida, F., Watanabe, K., Sato, A., Taguchi, K., Kakubari, K., Kitani, K., and Kamei, T. (1990) Comparative Effects of Simvastatin (MK-733) and Pravastatin (CS-514) on Hypercholesterolemia Induced by Cholesterol Feeding in Rabbits, *Biochim. Biophys. Acta 1042*, 365–373.
18. Nakamura, C.E., and Abeles, R.H. (1985) Mode of Interaction of β-Hydroxy-β-Methylglutaryl Coenzyme A Reductase with Strong Binding Inhibitors: Compactin and Related Compounds, *Biochemistry 24*, 1364–1376.

[Received December 4, 1993, and in final revised form July 6, 1995; Revision accepted August 22, 1995]

ANNOUNCEMENTS

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

For reference to journal articles:

• 1. Turner, E.M., Miller, E., and Smith, D.E. (1975) Temperature and Diet Effects on Trout, *J. Biol. Chem. 250,* 108–115.

For reference to books:

1. Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

For reference to multi-author books:

• 1. Turner, E.H. (1967) Temperature and Diet Effects on Trout, in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

For reference to theses:

1. Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

Manuscripts accepted for publication should be listed as:

• 1. Turner, E.H., Temperature and Diet Effects on Trout, *J. Biol. Chem.*, in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: All titles in References section should be capitalized. See examples above.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

AVAILABILITY OF PURIFIED OMEGA-3 FATTY ACIDS

Kilogram quantities of EPA and DHA ethyl ester capsules are available free of charge through the Fish Oil Test Materials Program, a joint effort of the National Oceanic and Atmospheric Administration and National Institutes of Health. These quality-assured and quality-controlled materials contain 94% EPA or 90% DHA. Ethyl esters of vegetable oils (olive, corn, and safflower) are available in capsule form as controls. Funded research projects studying the effect of

(continued on next page)

omega-3 fatty acids in health and disease, from all funding sources, are eligible to receive these capsules. For further information or to request a short application form, contact the Fish Oil Test Materials Program at 12501 Washington Ave., Rockville, MD 20852 (Phone: 301-443-9643; Fax: 301-594-0035).

CALENDAR

February 1996
Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

May 1996
XVIth Washington International Spring Symposium on Frontiers in Bioactive Lipids—'96, May 6–9, 1996, Washington, D.C., USA. Information: Dr. Jack Y. Vanderhoek, Chair—Symposium '96, Dept. of Biochemistry and Molecular Biology, The George Washington University, 2300 Eye St., N.W. RN 5530, Washington DC 20037-2337 USA (Phone: 202-994-2929; Fax: 202-994-8974; E-mail: jyvdh@gwis2.circ.gwu.edu.). Deadline for submission of abstracts: February 1, 1996.

July 1996
European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

July–August 1997
Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.

Purification and Characterization of a Cytochrome P450 Isozyme Catalyzing Lanosterol 14α-Demethylation (P450*14DM*) in Hamster Liver

Yoshio Sekigawa[a,1], Morio Fukuhara[a,*], Yoshiko Sonoda[b], and Yoshihiro Sato[b]

[a]Department of Pharmaceutical Sciences, National Institute of Public Health and [b]Department of Biochemistry, Kyoritsu College of Pharmacy, Tokyo 105, Japan

ABSTRACT: To characterize cholesterol synthesis in Syrian golden hamster, an isozyme of cytochrome P450, lanosterol 14α-demethylase (P450*14DM*), which catalyzes the initial step in the biosynthesis of cholesterol from lanosterol, was purified and its mode of induction by microsomal enzyme inducers was characterized. P450*450DM* was purified from hamster livers by chromatography using aminooctyl-Sepharose CL-4B, hydroxylapatite, DEAE-5PW, and CM-Sepharose CL-6B columns, to a specific content of 12.8 nmol/mg-protein. The purified protein displayed a single band on SDS–polyacrylamide gel electrophoresis with an apparent molecular weight of 52,000. The absorption spectra of the oxidized form of the purified protein showed a Soret peak at 417 nm in a low-spin state and a Soret peak of reduced CO-binding complex at 448 nm. In a reconstituted system, the purified protein catalyzed 14α-demethylation of 24,25-dihydrolanosterol (1.58 nmol/min/nmol-P450), although it did not show any activities toward testosterone and 7-ethoxyresorufin, marker substrates of other P450 families. Immunoblot analysis using an antibody against porcine P450*14DM*, which inhibited the activity of lanosterol 14α-demethylation in the hamster liver microsomes, demonstrated that the level of this isozyme protein was markedly decreased in dexamethasone-treated hamster livers. This was accompanied by a decrease in the enzyme activity. In contrast, the levels and the activity in the phenobarbital- and 3-methylcholanthrene-treated hamsters were almost equal to that in the untreated animals.

Lipids 30, 1067–1073 (1995).

It is well known that there are differences in hepatic cholesterol synthesis among mammalian species. Several studies disclosed significant quantitative differences in sterol balance among the species. For example, hepatic cholesterol metabolism differs between the rat and hamster. In general, activities of the rate-limiting enzyme in the biosynthesis of cholesterol (e.g., acyl-CoA/cholesterol acyltransferase and 3-hydroxy-3-methyl-glutaryl-CoA reductase) in the hamster are lower than in the rat (1). It has been reported that the hamster might offer a better animal model for examining human plasma lipoprotein profiles, lipoprotein metabolism, and liver cholesterol homeostasis than the rat (2–4).

In the metabolism of cholesterol, several steps are mediated by P450 isozymes. Marked species differences are known in mammalian cytochrome P450 (P450) involved in the activation of xenobiotics, including carcinogens. However, relatively few studies have been done on the species differences in P450 isozymes that metabolize endogenous substances, such as cholesterol, steroid hormones, and prostaglandins. Hamsters are known to have P450 isozymes with different properties from those of other animals (5–7). However, it is not fully known whether P450 isozymes in the hamster are similar to those of other species in metabolism of endogenous substances.

The initial step of cholesterol biosynthesis from lanosterol in yeast and mammals is the oxidative removal of the 14α-methyl group (C-32) from lanosterol, which is catalyzed by P450 enzyme, classified as P450*14DM* (P450 isozyme catalyzing lanosterol 14α-demethylation). The isozymes have been purified from yeast (8), rat liver (9,10), porcine liver (11), and human liver (12).

These earlier studies suggested that P450*14DM* in the hamster and other mammalian species might differ with respect to catalytic properties and regulation. However, little information is available on the mode of regulation of P450*14DM* in mammalian species or changes induced by xenobiotic treatment.

In the present study, we determined inducibility of P450*14DM* using immunoblot analysis and enzyme assay in Syrian golden hamsters treated with various inducers. We also purified P450*14DM* from Syrian golden hamster livers, characterized its spectral and catalytic properties, and tested its immunoreactivity with other P450 family proteins.

[1]Current address: Central Research Laboratories, Zeria Pharmaceutical Co., Saitama 360-01, Japan.

*To whom correspondence should be addressed at the Department of Pharmaceutical Sciences, National Institute of Public Health, Shirokanedai 4-6-1, Minato-ku, Tokyo 108, Japan.

Abbreviations: anti-P450*14DM*, anti-porcine P450*14DM* antibody; DHL, 24,25-dihydrolanosterol (lanost-8-3n-3β-ol); P450*14DM*, P450 isozyme catalyzing lanosterol 14α-demethylation; PAGE, polyacrylamide gel electrophoresis.

Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Materials. Aminooctyl Sepharose CL-4B and CM Sepharose CL-6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxylapatite and TSK-gel DEAE-5PW (150 × 21.5 mm i.d., particle size 13 μm) were obtained from Bio-Rad Laboratories (Richmond, CA) and Toso Co. (Tokyo, Japan), respectively. Emulgen 911 was a kind gift by Kao Co. (Tokyo, Japan). 24,25-Dihydrolanosterol (DHL) was prepared as described previously (11). Polyclonal antibodies against P450*14DM* protein were prepared as reported (12). Chemiluminescence reagent for Western blot was obtained from DuPont NEN (Boston, MA). The antibody to rat P450 2C11 was obtained from OXY gene (Dallas, TX). The antibodies to rat P450 2B1 and P450 4A1 were a kind gift from Dr. Omata, Department of Pharmacology, School of Medicine, Kurume University (Fukuoka, Japan). The antibodies to Syrian hamster P450 1A2, Chinese hamster P450 2A2, Syrian hamster P450 3A subfamilies, and porcine P450*14DM* were prepared in our laboratory. All other reagents used were of the highest quality available.

Treatment of animals and preparation of liver microsomes. Male Syrian golden hamsters and male Wistar rats (7–10 weeks old) were obtained from Nippon SLC (Hamamatsu, Japan). Animals were treated either with phenobarbital (60 mg/kg/d) for 4 d, 3-methylcholanthrene (25 mg/kg/d) for 3 d, or dexamethasone (100 mg/kg/d) for 3 d. These inducers were suspended in olive oil and injected intraperitoneally. Control animals received the vehicle only. Animals were killed one day after the last injection, and the livers were removed and homogenized in four volumes of 0.05 M Tris-HCl buffer (pH 7.4), containing 0.154 M KCl and 1 mM EDTA. The homogenates were centrifuged at 9,000 × *g* for 20 min, and then the supernatants were further centrifuged at 105,000 × *g* for 1 h to give microsomal pellets. The microsomes were resuspended in 0.05 M Tris-HCl buffer (pH 7.4), containing 0.154 M KCl, 1 mM EDTA, and 20% glycerol, and stored at −80°C until use.

*Purification of hamster P450*14DM. Hepatic microsomes from male Syrian golden hamsters treated with phenobarbital were suspended in 0.1 M potassium phosphate buffer (pH 7.4), which contained 20% glycerol, 1 mM EDTA, 1 mM DTT, and 0.7% cholic acid to produce final protein concentration of 2 mg/mL. The suspension was stirred gently for 60 min, and then centrifuged at 105,000 × *g* for 1 h. The supernatant was applied to an aminooctyl Sepharose CL-4B column (2.5 × 60 cm) previously equilibrated with 100 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol, 0.7% cholic acid, 1 mM EDTA, and 1 mM DTT. The column was washed with two volumes of 10 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol, 0.4% cholic acid, 1 mM EDTA, and 1 mM dithiothreitol (buffer A), and eluted with buffer A, containing 0.5% Emulgen 911. Eluted fractions were concentrated and dialyzed against 20 mM potassium phosphate buffer (pH 7.25), which contained 20% glycerol and 0.2% Emulgen 911 (buffer B). The resulting solution was applied to a hydroxylapatite column (5 × 5 cm) equilibrated with buffer B. The column was then washed with two volumes of buffer B with 60 mM and then with 90 mM potassium phosphate buffer (pH 7.25), containing 20% glycerol and 0.2% Emulgen 911, respectively. P450*14DM* was eluted with 200 mM potassium phosphate buffer (pH 7.25), which contained 20% glycerol and 0.2% Emulgen 911. Eluted fractions were concentrated and dialyzed against 5 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol and 0.4% Emulgen 911 (buffer C). The solution was applied to a high-performance liquid chromatography equipped with a preparative DEAE-5PW column (21.5 × 150 mm i.d.) equilibrated with buffer C. After the pass through fraction was eluted, a 20–180 mM linear gradient of potassium phosphate buffer was applied. The fractions containing P450 showing immunoreactivity to anti-porcine P450*14DM* antibodies were concentrated and dialyzed with 20 mM potassium phosphate buffer (pH 6.5), containing 20% glycerol and 0.2% Emulgen 911 (buffer D). The resulting solution was applied to a CM Sepharose CL-6B column (1.5 × 5 cm) equilibrated with buffer D. The column was washed with buffer D, and successively eluted with buffer D, containing 60, 90, and 150 mM potassium phosphate to give a final P450*14DM* fraction.

Purification of NADPH-cytochrome c reductase. NADPH-cytochrome c reductase was purified from hamster livers, according to the procedure described by Yasukochi and Masters (13).

Gel electrophoresis and immunoblot analysis. SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (14), and separated proteins were visualized by silver staining. For immunoblot analysis, the separated proteins were transferred onto nitrocellulose membranes or polyvinylidene difluoride membranes, which were then developed using the antibodies raised against various P450 family proteins and detected by using the peroxidase–diaminobenzidine–tetrahydrochloride method (15) or by luminescence-monitored enzyme immunoassay (16).

*Characterization of the purified P450*14DM. The spectra of the purified P450*14DM* were measured by the method of Omura and Sato (17). The apparent molecular weight of the purified P450*14DM* was determined by SDS–PAGE with 10% acrylamide. The catalytic activities of the purified protein were determined using a reconstituted monooxygenase system consisting of the purified P450*14DM* (10–300 pmol), dilauroyl-L-α-phosphatidylcholine, NADPH-generating system, and NADPH-cytochrome c reductase (1 unit). The catalytic activity was measured toward DHL (23 μM of substrate concentration) (11), testosterone (0.25 mM) (18), and 7-ethoxyresorufin (0.3 mM) (19).

Assay of cholesterol synthesis from DHL using hepatic microsomes. The activity of DHL 14α-demethylation was determined by the amounts of cholesterol produced from DHL. Microsomes (2 μg protein) were incubated at 37°C for 30 min at the presence of [24,25-$^{3}H_{2}$]-24,25-DHL (0.37 kBq/mmol,

50 μM) and cofactors according to the method of Trzaskos *et al.* (20). Incubations were started by the addition of DHL. After incubation, methanol and potassium hydroxide were added to a final concentration of 50 and 10%, respectively. The mixture was heated at 70°C for 1 h, then extracted with dichloromethane, washed with water, and dried under nitrogen. The resulting residues were dissolved in 200 μL of dichloromethane, and were subjected to thin-layer chromatography. The band corresponding to cholesterol was collected, and the radioactivity was measured by a scintillation counter.

Protein assay. Protein contents were determined using Lowry's method (21) with bovine serum albumin as standard.

RESULTS

*Effect of anti-porcine P450*14DM *antibody (anti-P450*14DM*) on DHL 14α-demethylase activity in hamster livers.* To ascertain the affinity of the anti-P450*14DM* of pig (11) to hamster P450*14DM*, the inhibition of DHL 14α-demethylation activity of the hamster by the antibody was examined in the fractions containing hamster P450*14DM* obtained from the aminooctyl Sepharose CL-4B column. Anti-P450*14DM* inhibited DHL 14α-demethylation of hamster enzyme in a concentration-dependent manner (Fig. 1).

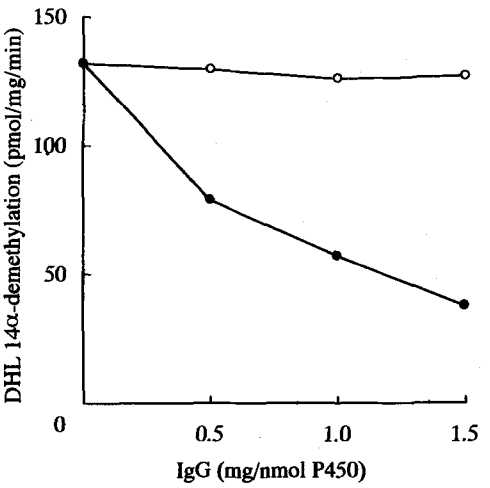


FIG. 1. Inhibitory effect of anti-porcine P450 isozyme catalyzing lanosterol 14α-dimethylation (P450*14DM*) antibody on 24,25-dihydrolanosterol (lanost-8-en-3β-ol) (DHL) 14α-demethylase activities in hamster hepatic microsomes. Hamster hepatic microsomes were incubated with the IgG from anti-sera containing antibodies against porcine P450*14DM* or from preimmune sera prior to the assay of DHL 14α-demethylase. Each point represents the mean of two determinations. (●), anti-porcine P450*14DM*; (○); preimmune sera.

*Purification of hamster P450*14DM. P450*14DM* was purified from hepatic microsomes of phenobarbital-treated hamsters, and a representative result of the purification of P450 is summarized in Table 1. Following solubilization with cholate and fractionation with aminooctyl Sepharose CL-4B column chromatography, the fraction containing P450*14DM* was further fractionated on a hydroxylapatite column. As shown in Figure 2, hydroxylapatite chromatography gave several peaks, which were examined by immunoblot analysis using anti-P450*14DM* (Fig. 3). Anti-P450*14DM* crossreacted strongly to the third fraction (HA-3), which was then collected. As shown in Figure 4, in the final purification step using CM-Sepharose CL-6B chromatography, three peaks were eluted from the column. DHL 14α-demethylase activity was detected in the second (CM-1) and the third (CM-2) fractions, and the former showed higher DHL 14α-demethylase activity than the latter (data not shown). The "CM-1" was collected and designated as P450*14DM* preparation. The specific P450 content of the final preparation was 12.8 nmol/mg-protein.

*Physicochemical and immunochemical properties of the purified hamster P450*14DM. The purified P450*14DM* demonstrated a single protein band upon SDS–PAGE and on immunoblot analysis using anti-P450*14DM*. The apparent molecular weight of P450*14DM* was estimated to be 52,000

TABLE 1
Purification of P450*14DM* from Hepatic Microsomes of Phenobarbital-Treated Hamster Livers[a]

Purification step	Protein (mg)	P450 Total (nmol)	P450 Specific recovery (nmol/mg)	P450 Specific recovery (%)	DHL 14α-demethylation (nmol/min/mg protein)
Microsomes	833	1532	1.8	100.0	n.d.[d]
Solubilized microsomes	584	1157	2.0	75.5	n.d.
Aminooctyl Sepharose CL-4B[b]	272	810	3.0	52.9	0.13
Hydroxylapatite	73	292	4.0	19.1	n.d.
DEAE-5PW[c]	11	77	6.7	5.0	n.d.
CM Sepharose CL-6B[b]	2	24	12.8	1.6	20.0

[a]P450*14DM*, P450 isozyme catalyzing lanosterol 14α-demethylation; DHL, 24,25-dihydrolanosterol (lanost-8-en-3β-ol).
[b]From Pharmacia (Uppsala, Sweden).
[c]From Toso (Tokyo, Japan).
[d]n.d., Not determined.

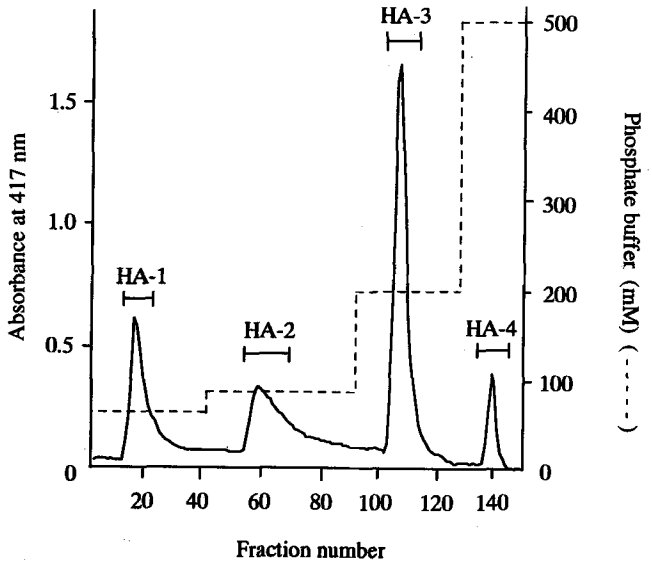


FIG. 2. Elution profile of the P450 fraction from hydroxylapatite column. P450 isozymes were separated on a hydroxylapatite column using a stepwise concentration of a potassium phosphate as described in the Materials and Methods section. One fraction contains 5 mL of eluate.

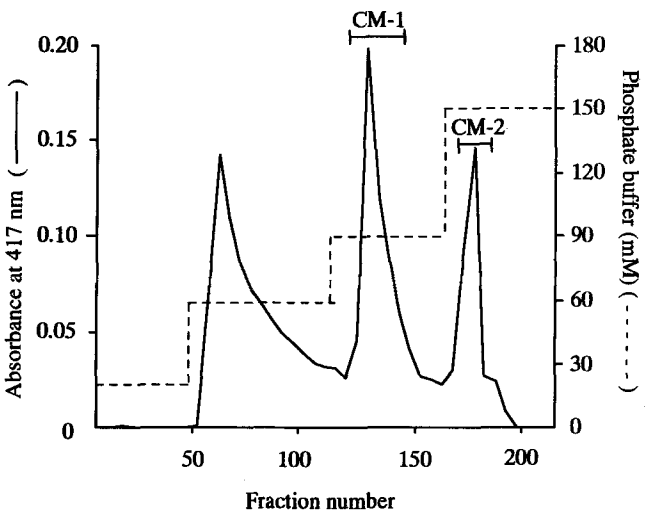


FIG. 4. Elution profile of P450 from CM Sepharose CL-6B (Pharmacia, Uppsala, Sweden). P450 isozymes were separated on a CM Sepharose CL-6B column using a stepwise concentration of potassium phosphate as described in the Materials and Methods section. One fraction contains 5 mL of eluate.

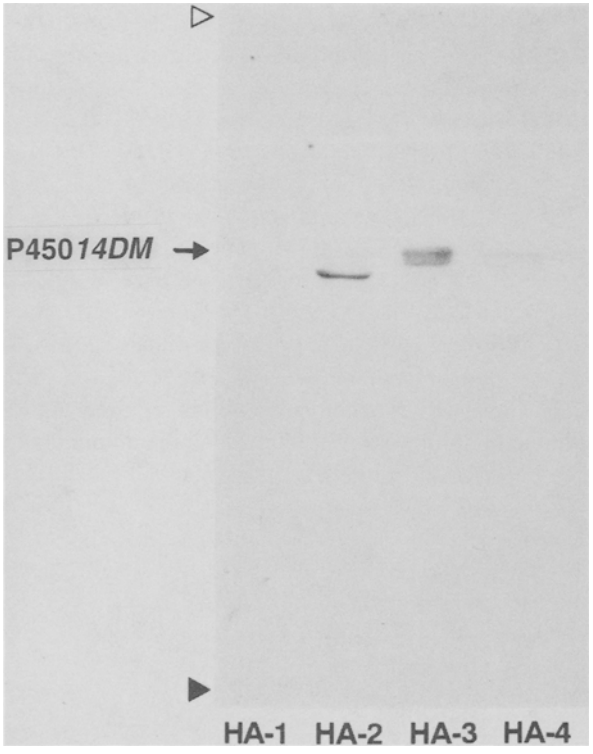


FIG. 3. Immunoblot analysis of the eluted fractions from hydroxylapatite column. Numbers of the individual samples corresponded to those of separated fractions displayed in Figure 2. Each sample (5 μg of protein) was submitted to SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and stained using the antibody against porcine P450*14DM* and peroxidase–diaminotetrahydrochloride.

(Fig. 5A). In immunoblot analysis, the antibody against P450*14DM* recognized two protein bands in hamster hepatic microsomes, and the purified enzyme corresponded to the band with a higher molecular weight (Fig. 5B).

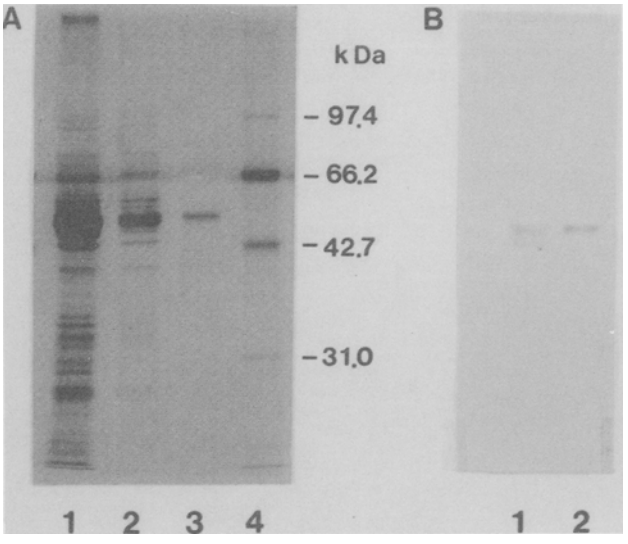


FIG. 5. SDS–polyacrylamide gel electrophoresis (PAGE) (A) and immunoblot analysis (B) of the purified P450*14DM* and hepatic microsomes of phenobarbital-treated hamsters. Panel A: hepatic microsomes from phenobarbital-treated hamsters (lane 1, 1 μg protein), solubilized hepatic microsomes from phenobarbital-treated hamsters (lane 2, 1 μg protein), purified P450*14DM* (lane 3, 0.05 μg protein), and molecular weight standard mixture (lane 4) were electrophoresed on 10% acrylamide gels and stained with silver. Panel B: microsomes from phenobarbital-treated hamster livers (lane 1, 20 μg protein) and purified P450*14DM* (lane 3, 1 μg protein) were submitted to SDS–PAGE, transferred to nitrocellulose membranes, and stained using the antibody against porcine P450*14DM* and peroxidase–diaminobenzidine–teterahydrochloride.

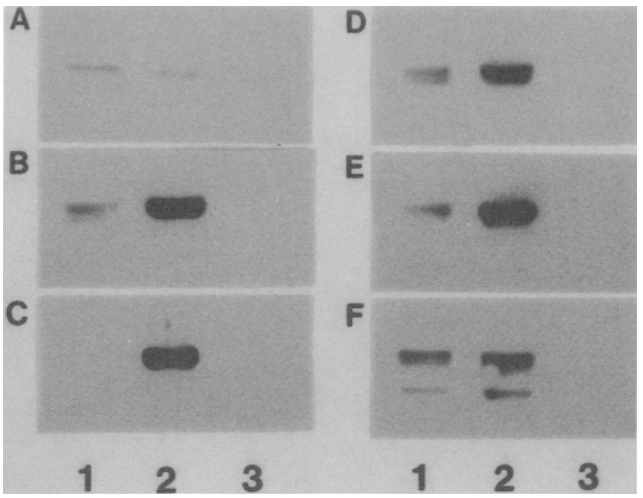


FIG. 6. Imunoblot analysis of the purified P450*14DM* using antibodies against P450 isozymes. P450 samples were submitted to SDS–PAGE, transferred to nitrocellulose membranes, and stained immunochemically using the antibody against Syrian hamster P450 1A2 (panel A), Chinese hamster P450 2A2 (panel B), rat P450 2B1 (panel C), rat P450 2C11 (panel D), Syrian hamster P450 3A (panel E), and rat P450 4A1 (panel F). Lanes 1 and 2 contain hepatic microsomes (5 μg protein) from untreated and phenobarbital-treated hamsters, respectively. Lane 3 contains purified P450*14DM* (0.1 μg protein) and peroxidase–luminol–hydrogen peroxide reaction. Abbreviations as in Figures 1 and 5.

To ascertain the homogeneity of the purified hamster P450*14DM*, we examined the crossreactivity of the protein with the antibodies against other P450 isozymes (P450 1A2, 2A2, 2B1, 2C11, 3A, and 4A1) by immunoblot analysis. As shown in Figure 6, the purified hamster P450*14DM* did not react with these antibodies.

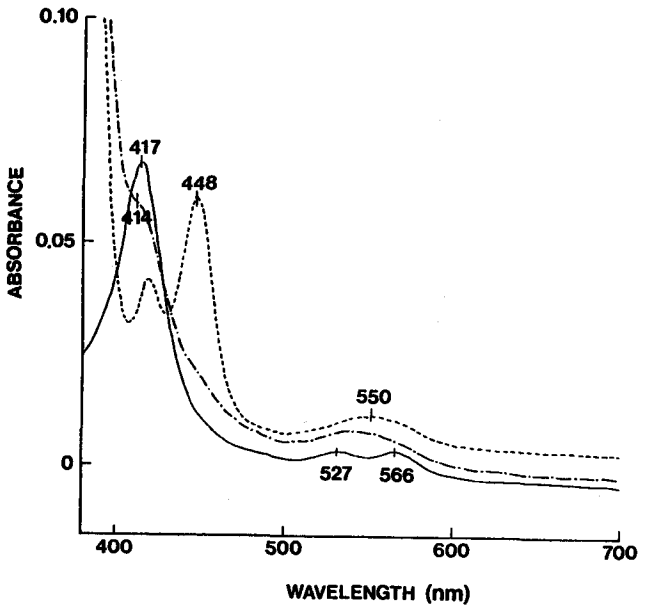


FIG. 7. Absolute spectra of the purified P450*14DM*. The concentration of P450*14DM* was 0.4 nmol/mL–0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol. Oxidized, —; dithionite-reduced, –·–; reduced/CO-complex, ----. Abbreviation as in Figure 1.

TABLE 2
Effects of Various Xenobiotic Inducers upon Cholesterol Synthesis from DHL and Microsomal P450 Level

Inducers	Cholesterol synthesis from DHL[a] (pmol/min/mg protein)	P450 (nmol/mg protein)
Control (olive oil)	132 ± 5	0.907 ± 0.103
Phenobarbital	173 ± 16	1.591 ± 0.071[b]
3-Methylcholanthrene	122 ± 5	1.540 ± 0.078[b]
Dexamethasone	27 ± 1[b]	0.574 ± 0.037

[a]This activity represents DHL 14α-demethylation activity expressed as amounts of cholesterol converted from DHL per unit of microsomal protein. Data are expressed as means ± SE for three animals with statistical significance at [b]$P < 0.01$ vs. control (Student's *t*-test). Abbreviation as in Table 1.

The absolute spectra of the purified P450*14DM* showed that the oxidized form of this enzyme had a Soret band at 417 nm, the α bands at 527 and 566 nm (Fig. 7). This indicates that the P450*14DM* was of low-spin ferric P450. The difference absorption spectra of reduced CO-complex showed a maximum at 448 nm.

*Catalytic properties of purified P450*14DM. The catalytic activities of the purified P450*14DM* toward testosterone and 7-ethoxyresolfin were examined in a reconstituted system. The purified enzyme showed DHL 14α-demethylase activity (1.58 nmol/min/nmol-P450), but it did not show any detectable 7-ethoxyresorufin *O*-deethylase or testosterone hydroxylase activity.

*Effects of various xenobiotics upon P450*14DM *induction.* To determine the induction mode of P450*14DM*, hamsters were treated with phenobarbital, 3-methylcholanthrene, and dexamethasone and the level of P450*14DM* protein and the changes in the conversion activity of DHL to cholesterol in hepatic microsomes were determined instead of measuring DHL 14α-demethylase. DHL is known to be converted to its final product, cholesterol, when incubated with hepatic microsomes (20). To determine the activity of DHL 14α-demethylase using hepatic microsomes, the addition of two inhibitors in the reaction mixture that block the following reactions after DHL 14α-demethylation is required. As we did not have the inhibitors, we determined the amounts of cholesterol converted from DHL. As shown in Table 2, the conversion rate of DHL to cholesterol in hepatic microsomes decreased by treatment with dexamethasone, but it was not changed by treatment with phenobarbital with 3-methylcholanthrene. The level of P450*14DM* protein analyzed by immunoblotting in hepatic microsome was markedly decreased by treatment with dexamethasone (Fig. 8). These results show that conversion activity of DHL to cholesterol parallels protein levels of P450*14DM* in hepatic microsomes of hamster treated with inducers.

DISCUSSION

In the present study, we purified to apparent homogeneity P450*14DM* from phenobarbital-treated hamster livers. The purified protein was confirmed to be P450*14DM*, as it cross-

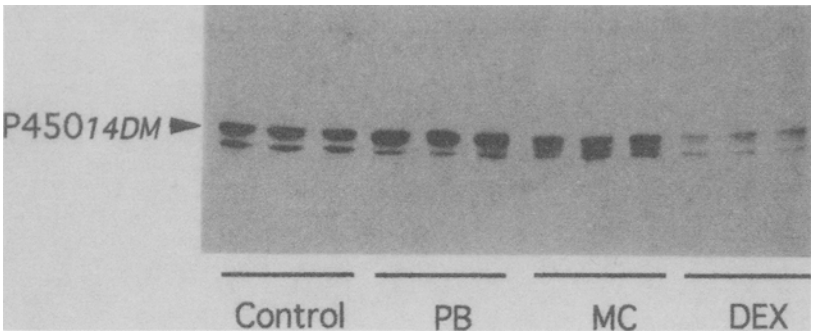


FIG. 8. Inducibility of P450*14DM* in hamster livers by phenobarbital, 3-methylcholanthrene, and dexamethasone. Immunoblot analysis was done on the hepatic microsomes from hamsters treated with phenobarbital (PB), 3-methylcholanthrene (MC), and dexamethasone (DEX). Each well contains 2 μg of microsomal protein with the exception of purified hamster P450*14DM*. The proteins, separated by SDS–PAGE, were transferred to polyvinylidene difluoride membranes, and stained using the antibody against porcine P450*14DM* and peroxidase–luminol–hydrogen peroxide reaction. Abbreviations as in Figures 1 and 5.

reacted with anti-P450*14DM* and showed a high DHL 14α-demethylase activity. As shown in Figure 1, anti-porcine P450*14DM* antibody (anti-P450*14DM*) dose-dependently inhibited the DHL 14α-demethylase activity in hamster hepatic microsomes. This indicates that hamster P450*14DM* is immunologically related to porcine P450*14DM* and that anti-P450*14DM* can be used to study hamster P450*14DM*.

In hamster livers, two bands were detected by immunoblot analysis using anti-P450*14DM*. The purified enzyme showing DHL 14α-demethylase activity corresponded to the upper band of the two in the hepatic microsomes of hamster (Fig. 5B). We also recognized two bands in the rat, and the lower band of the two did not show any DHL 14α-demethylase activity (10). P450*14DM* has been purified from hepatic microsomes of pig (11), human (12), and rat (10). Catalytic properties, including substrate specificity of the purified P450*14DM*, have not been fully characterized. We examined catalytic properties of hamster P450*14DM* toward the substrates other than DHL. However, it did not show any catalytic activities toward testosterone and 7-ethoxyresorufin that are specific to other P450 isozymes, such as P450 2A, P450 2B, and P450 3A (22). These suggest that substrate specificity of P450*14DM* might be very narrow.

As for the induction mode of P450*14DM* in the liver, induction of P450*14DM* by xenobiotic treatment was previously reported by measuring the changes in lanosterol 14α-demethylase activity in rat liver (20). In the present study, we examined the inducibility of P450*14DM* in hamster liver by immunoblot analysis using anti-P450*14DM* and by measuring the enzyme activity (Table 2). Of the inducers examined, dexamethasone-treatment markedly decreased the level of P450*14DM* in hepatic microsomal P450 and DHL 14α-demethylase activity. On the other hand, phenobarbital- and 3-methylcholanthrene-treatment did not affect the level of P450*14DM*. It is well established that, in rat liver, dexamethasone causes a significant increase in P450 content (23). However, we demonstrated that in the hamster, treatment with dexamethasone dramatically decreased the amounts of hepatic microsomal P450 and also the level of P450 3A subfamily protein (6). These findings indicated that in the hamster, dexamethasone may decrease most of P450 isozymes, including P450*14DM*.

Little information is available, so far, on the properties of hamster P450*14DM*. In the present study, we have characterized the physicochemical and catalytic properties of the purified P450*14DM* and demonstrated differences in the properties of P450*14DM* of the hamster from those of the rat, notably in the induction mode. Further studies on P450*14DM* of hamster livers with respect to the induction mechanism and cDNA structure are needed to explain species differences in cholesterol synthesis.

REFERENCES

1. Suckling, K.E., and Stange, E.F. (1985) Role of Acyl CoA: Cholesterol Acyltransferase in Cellular Cholesterol Metabolism, *J. Lipid Res. 26*, 647–671.
2. Spady, D.K., Meddings, J.B., and Dietschy, J.M. (1986) Kinetic Constants for Receptor-Dependent and Receptor-Independent Low Density Lipoprotein Transport in the Tissues of the Rat and Hamster, *J. Clin. Invest. 77*, 1474–1481.
3. Spady, D.K., Turley, S.D., and Dietschy, J.M. (1985) Rates of Low Density Lipoprotein Uptake and Cholesterol Synthesis Are Regulated Independently in the Liver, *J. Lipid Res. 26*, 465–472.
4. Spady, D.K., and Dietschy, J.M. (1983) Sterol Synthesis *in vivo* in 18 Tissues of the Squirrel Monkey, Guinea Pig, Rabbit, Hamster, and Rat, *J. Lipid Res. 24*, 303–315.
5. Sunouchi, M., Fukuhara, M., Ohno, Y., and Takanaka, A. (1988) Effects of Various Inducers on the Activities of Cytochrome P-450-Mixed Function Oxidase and Aflatoxin B_1 Activation in Microsomes of Hamster Livers, *J. Toxicol. Sci. 13*, 193–204.
6. Antignac, E., and Fukuhara, M. (1992) Identification of a Constitutive Form of Cytochrome P-450 with High Activity Towards Macrolide Antibiotics in Syrian Golden Hamster Liver, *Res. Commun. Chem. Path. Pharmacol. 76*, 43–51.
7. Antignac, E., Fukuhara, M., and Kimura, M. (1993) Effects of Dexamethasone on the Hepatic Cytochrome P450IIIA Subfam-

ily in Two Hamster Strains *Mesocricetus auratus* and *Cricetus griseus*, *Comp. Biochem. Physiol. 104C*, 433–437.
8. Yoshida, Y., and Aoyama, Y. (1984) Yeast Cytochrome P-450 Catalyzing Lanosterol 14α-Demethylation: I. Purification and Spectral Properties, *J. Biol. Chem. 259*, 1655–1660.
9. Trzaskos, J., Kawata, S., and Gaylor, J.L. (1986) Microsomal Enzymes of Cholesterol Biosynthesis: Purification of Lanosterol 14α-Methyl Demethylase Cytochrome P-450 from Hepatic Microsomes, *J. Biol. Chem. 261*, 14651–14657.
10. Endo, M., Sonoda, Y., Ishida, K., Sato, Y., Sekigawa, Y., and Fukuhara, M. (1994) Characterization of Catalytic Properties and Expression of Cytochrome $P\text{-}450_{14DM}$, Lanosterol 14α-Demethylase, in Rats, *Res. Commun. Mol. Pathol. Pharmacol. 85*, 279–289.
11. Sono, H., Sonoda, Y., and Sato, Y. (1991) Purification and Characterization of Cytochrome $P\text{-}450_{14DM}$ (Lanosterol 14α-Demethylase) from Pig Liver Microsomes, *Biochim. Biophys. Acta. 1078*, 388–394.
12. Sonoda, Y., Endo, M., Ishida, K., Sato, Y., Fukusen, N., and Fukuhara, M. (1993) Purification of a Human Cytochrome P-450 Isozyme Catalyzing Lanosterol 14α-Demethylation, *Biochim. Biophys. Acta. 1170*, 92–97.
13. Yasukochi, Y., and Masters, B.S.S. (1976) Some Properties of a Detergent-Solubilized NADPH-Cytochrome c (Cytochrome P-450) Reductase Purified by Biospecific Affinity Chromatography, *J. Biol. Chem. 251*, 5337–5344.
14. Laemmli, U.K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, *Nature 227*, 680–685.
15. Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, H.V., and Kaminsky, L.S. (1982) Purification and Characterization of Liver Microsomal Cytochromes P-450: Electrophoretic, Spectral, Catalytic, and Immunochemical Properties and Inducibility of Eight Isozymes Isolated from Rats Treated with Phenobarbital or β-Naphthoflavone, *Biochemistry 21*, 6019–6030.
16. Thorpe, G.H.G., Kricka, L.J., Mosely, S.B., and Whitehead, T.P. (1985) Phenols as Enhancers of the Chemiluminescent Horseradish Peroxidase-Luminol-Hydrogen Peroxide Reaction: Application in Liminescence-Monitored Enzyme Immunoassays, *Clin. Chem. 31*, 1335–1341.
17. Omura, T., and Sato, R. (1964) The Carbon Monooxide-Binding Pigment of Liver Microsomes. 1. Evidence for Its Hemoprotein Nature, *J. Biol. Chem. 239*, 2370–2378.
18. Yamazoe, Y., Shimada, M., Murayama, N., and Kato, R. (1987) Suppression of Levels of Phenobarbital-Inducible Rat Liver Cytochrome P-450 by Pituitary Hormone, *J. Biol. Chem. 262*, 7423–7428.
19. Burke, M.D., and Mayer, R.T. (1975) Inherent Specificities of Purified Cytochromes P-450 and P-448 Toward Biphenyl Hydroxylation and Ethoxyresorufin Deethylation, *Drug Metab. Disp. 3*, 245–253
20. Trzaskos, J.M., Bowen, W.D., Shafiee, A., Fisher, R.T., and Gaylor, J.L. (1984) Cytochrome P-450-Dependent Oxidation of Lanosterol in Cholesterol Biosynthesis: Microsomal Electron Transport and C-32 Demethylation, *J. Biol. Chem. 259*, 13402–13412.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
22. Gonzalez, F.J. (1989) The Molecular Biology of Cytochrome P450s, *Pharmacol. Rev. 40*, 243–288.
23. Heumen, D.M., Gallagher, E.J., Barwick, J.L., Elshourbagy, N.A., and Guzelian, P.S. (1982) Immunochemical Evidence for Induction of a Common Form of Hepatic Cytochrome P450 in Rat Treated with Pregnenolone 16α-Carbonitrile or Other Steroidal or Nonsteroidal Agents, *Mol. Pharmacol. 21*, 753–760.

[Received April 12, 1995, and in final revised form September 8, 1995; Revision accepted October 31, 1995]

Multiple Forms of the Enzyme Glycerophosphodiesterase Are Present in Human Brain

Brian M. Ross[a,*], Allan L. Sherwin[b], and Stephen J. Kish[a]

[a]Human Neurochemical Pathology Laboratory, Clarke Institute of Psychiatry, Toronto, Canada and [b]Montreal Neurological Institute, Montreal, Canada

ABSTRACT: Brain levels of glycerophosphodiesters, including glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE), are altered in many human central nervous system disorders. Although much information is available on the enzymes responsible for the formation of these phospholipid metabolites, little information is known regarding their catabolism, by glycerophosphodiesterases, in human brain. In both autopsied and biopsied temporal cortex, a phosphocholine-producing glycerophosphodiesterase activity was observed. In the presence of 1 mM EDTA, the enzyme possessed a pH optimum of 9.0, while the addition of 5 mM zinc acetate shifted the pH optimum to 10.5. When assayed at pH 9.0 in the absence of zinc acetate, the K_m and V_{max} were 104 ± 2 μM and 77 ± 18 nmol/h/mg protein, respectively, while assaying at pH 10.5 in the presence of 5 mM zinc acetate yielded a K_m of 964 ± 56 μM, and a V_{max} of 534 ± 114 nmol/h/mg protein. Furthermore, whereas submillimolar concentrations of zinc acetate stimulated the activity of the enzyme in a dose-dependent manner when assayed at pH 10.5 (EC_{50} = 20.3 ± 3.0 μM), this did not result in a reciprocal inhibition of glycerophosphocholine phosphodiesterase (GPC PD) activity when assayed at a more acidic pH. This may suggest that human brain contains two phosphocholine-producing GPC PD activities, differentiable by their sensitivity to zinc ions. An activity capable of hydrolyzing GPE to form phosphoethanolamine could not be detected in either biopsied or autopsied brain. However, a choline/ethanolamine-producing glycerophosphodiesterase activity could be readily detected in biopsied, but not autopsied brain. This novel enzyme possessed a neutral pH optimum and was dependent upon divalent cations for activity. In conclusion, human brain contains at least two different glycerophosphodiesterases, a phosphocholine, and a choline/ethanolamine-producing activity, only one of which can be detected in autopsied tissue. The results of previous studies measuring brain glycerophosphodiesterase activity in degenerative brain conditions may need to be reevaluated in the light of these observations.

Lipids 30, 1075–1081 (1995).

Glycerophosphodiesters (GPD), for example, glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE), are abundant catabolites of brain membrane phosphoglycerides formed by the sequential actions of phospholipase A and lysophospholipase (1). Recent studies have shown altered brain GPD levels in several central nervous system disorders. For example, *in vivo* phosphorus-31 nuclear magnetic resonance spectroscopy studies suggest increased brain levels of GPD in schizophrenia (2), autism (3), and Alzheimer's disease (4). This is supported by the post mortem finding of increased GPC concentration in the brains of patients with Alzheimer's disease (5) and olivopontecerebellar atrophy (6). Although these changes have frequently been attributed to altered rates of phosphoglyceride breakdown in these disorders, they also may be explained by changed activity of the GPC catabolizing enzyme, GPC phosphodiesterase (GPC PD). Previous investigations of this enzyme in animal brain have show it to be a family of at least three different types, two of which catalyze the formation of glycerol and phosphocholine, while the other yields glycerol phosphate and choline as products (7–9). However, no enzyme capable of hydrolyzing GPE, another major brain GPD, has been identified. Furthermore, studies of these enzymes in human brain have not defined or characterized the forms which are present (5,10,11). Since GPC is also of considerable interest, due to its possible therapeutic potential in Alzheimer's disease (12), and its enhancement of choline acetyl transferase activity (13), it is now timely to investigate the enzyme responsible for its catabolism in human brain, and to examine the characteristics of the subtypes present.

MATERIALS AND METHODS

Materials. Glycerophospho[^{3}H]choline and glycerophospho-[^{14}C]ethanolamine were synthesized from 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[methyl-1-^{3}H]choline and 1,2-dioleoyl-*sn*-glycero-3-phospho-[2-^{14}C]ethanolamine (Amersham, Oakville, Ontario, Canada), by alkaline alcoholysis (14). The identity of the synthesized compounds was confirmed as described (14). Radiochemical purity was greater than 98.5%, the remainder being composed of choline (ethanolamine) phosphate and choline (ethanolamine) as as-

*To whom correspondence should be addressed at Human Neurochemical Pathology Laboratory, Clarke Institute of Psychiatry, 250 College St., Toronto, Ontario, M5T 1R8 Canada.

Abbreviations: GPC, glycerophosphocholine, GPC PD, glycerophosphocholine phosphodiesterase; GPD, glycerophosphodiester; GPE, glycerophosphoethanolamine; TLC, thin-layer chromatography; Zn-GPC PD, zinc-sensitive GPC PD.

Copyright © 1995 by AOCS Press

sessed by thin-layer chromatography (TLC). The concentration, as deduced from the specific activity of the starting compounds, was confirmed by phosphate assay (15). TLC plates were purchased from Whatman (Chromatographic Specialties, Brockville, Toronto, Canada), and Dowex ion exchange resin from Biorad (Mississauga, Toronto, Canada). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), and were of the highest grade commercially available.

Brain tissue. Temporal cortices (Brodmann area 21) from the brains of four neurologically normal subjects of age 55 ± 14 yr (mean ± SEM) and post mortem interval 8 ± 1 h were used throughout this study. The brains had been frozen at autopsy and stored at −80°C. Biopsied temporal cortex, frozen immediately after surgical excision, was obtained from three individuals (age 25 ± 3 yr) who had undergone temporal lobectomy for epileptic seizures. The biopsied tissue was from nonepileptogenic regions as determined by depth recording electrography. All tissue was free of gross infarct and tumors, and autopsied tissue did not show any indication of neurodegenerative disease as assessed by neuropathological examination.

GPC PD assay. Brain tissue was dissected free of white matter and visible blood vessels, and sonicated for 15 s on ice using a Bonwell Biosonics sonicator (70% power) in 10 vol of 1 mM EDTA, 1 mM EGTA, and 50 mM HEPES/sodium hydroxide, pH 7.4. Aliquots containing 50 µg of protein were added to a buffer (total volume 200 µL) containing [^{3}H]-GPC (100,000–200,000 dpm), the exact reaction conditions for each experiment is described in the Results section. Boiled tissue homogenates were used as "blank" reactions; all samples were assayed in duplicate. The reaction was allowed to proceed for 45 min at 37°C before being terminated by the addition of 800 µL of chloroform/methanol (1:2). Phase separation was accomplished by the sequential addition of 200 µL chloroform and 200 µL water (16). The aqueous phase was removed, and the organic phase reextracted with 800 µL water. The combined aqueous phases were applied to 1 mL Dowex 50W-X8 (H^+) in a total volume of 4.7 mL. The columns were washed with 5 mL water to elute GPC, followed by 2 × 5 mL water to elute phosphocholine, and finally after washing with a further 5 mL water, choline was eluted with 3 mL of 3 M hydrochloric acid (14,17). Radioactivity in each fraction was determined by liquid scintillation spectroscopy. Elution volumes were determined using authentic radiolabelled standards. In some experiments, the aqueous phases were dried by vacuum centrifugation, resuspended in 30 µL water containing 200 µg GPC, 200 µg phosphocholine, and 40 µg choline, applied to silica gel TLC plates (Whatman LK5D), and developed in methanol/0.9% sodium chloride/30% ammonium hydroxide (70:50:5, by vol) (16). Compounds were visualized by staining with iodine vapor, scraped into 1 mL water and radioactively quantified. The TLC separation gave quantitatively similar results to the ion exchange method and, due to the lower reaction blanks when choline release was being investigated (approx. 800 dpm using ion exchange columns compared to 150 dpm using TLC), routinely was used to detect the production of free base. Similarly, when GPE was used as substrate, TLC separation was also used, with cold GPE (10 µg), phosphoethanolamine (10 µg), and ethanolamine (5 µg) being added as standards, the plate developed in methanol/0.5% sodium chloride/30% ammonium hydroxide (50:50:5), and the compounds visualized with ninhydrin.

Protein concentration was determined by the Biorad protein assay according to the manufacturers' instructions.

RESULTS

Phosphocholine-producing enzymes. The GPC PD activity of autopsied human temporal cortex catalyzed the hydrolysis of GPC to yield phosphocholine as the major product. The production of phosphocholine was linear with respect to time for at least one hour, and with respect to protein up to at least 150 µg homogenate protein (data not shown). The characteristics of GPC hydrolysis were dependent upon the ionic conditions used. Thus, in the presence of 1 mM EDTA or 5 mM calcium chloride, GPC PD activity exhibited a single pH optimum of 9.0, while the addition of 5 mM zinc acetate caused the pH

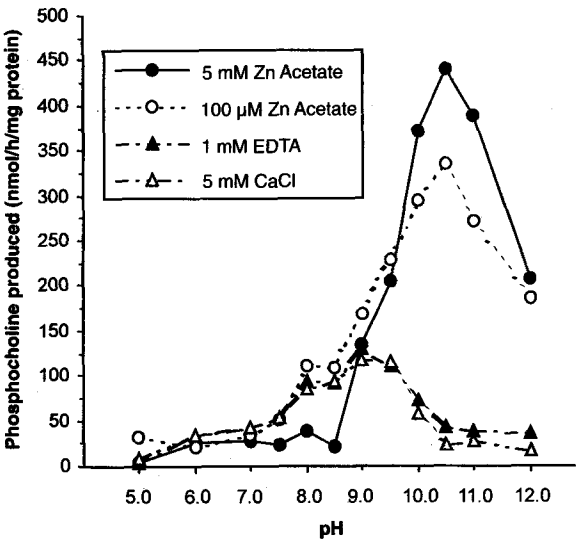


FIG. 1. Effect of assay pH upon the phosphocholine-producing glycerophosphocholine phosphodiesterase (GPC PD) activity of autopsied human brain. Activity was assayed using 5 mM [^{3}H]glycerophosphocholine as substrate under varying pH in the presence of 5 mM or 100 µM zinc acetate, 5 mM calcium chloride, or 1 mM EDTA as indicated. The assay buffer also contained 1 mM levamisole, and 12.5 µM EDTA, and 12.5 µM EGTA contributed by the homogenization buffer. The buffers used at each pH were: pH 5 and 6, 100 mM sodium acetate/acetic acid, pH 7–8, 100 mM HEPES/sodium hydroxide and pH 8.5–11, and 100 mM glycine/sodium hydroxide. Data represent the mean activity of three human temporal cortices analyzed in independent experiments. For reasons of clarity, error bars are not shown; average SEM was 14% as a percentage of mean activity. The rather high error values in this and other figures are not a consequence of high intra-assay variability, but are due to significantly different values of GPC PD activity being present in each brain. However, in all experiments, each brain examined yielded qualitatively similar results.

optimum to change to 10.5 (Fig. 1). Indeed, the addition of zinc acetate caused an approximately 10-fold stimulation of activity at pH 10.5. Furthermore, while the addition of 5 mM zinc acetate did not alter GPC PD activity when assayed at pH 9.0, it was inhibited markedly compared to zinc-free conditions at the slightly more acidic pH of 8.5. However, reducing the concentration of zinc acetate to 100 μM resulted in no inhibition relative to zinc-free conditions being observed at pH 8.5 (Fig. 1). To further investigate these two effects, namely zinc-mediated stimulation of GPC PD activity at pH 10.5, and zinc-mediated inhibition at pH 8.5, GPC PD activity was measured in the presence of varying concentrations of zinc acetate (Fig. 2). At pH 8.5, GPC PD activity was unaffected by zinc acetate concentrations below 1 mM, although higher concentrations caused inhibition, in agreement with Figure 1. In contrast, at pH 10.5, GPC hydrolysis was stimulated by zinc acetate in a dose-dependent manner (EC_{50} = 20.7 ± 3.0 μM; n = 3), with the highest activity occurring in the presence of 5 mM zinc acetate (Fig. 2).

Further investigations, in which the enzyme was assayed at either pH 8.5 in the presence of 1 mM EDTA, or pH 10.5 in the presence of 5 mM zinc acetate, showed that GPC hydrolysis exhibited Michaelis-Menton kinetics (Fig. 3), but with different K_m and V_{max} values under each condition: using 1 mM EDTA/pH 9.0 the K_m and V_{max} were 104 ± 2 μM and 77 ± 18 nmol/h/mg protein, respectively (by Eadie-Hofstee transformation), while in the presence of 5 mM zinc acetate/pH 10.5, K_m and V_{max} were 964 ± 56 μM and 534 ± 114 nmol/h/mg protein, respectively.

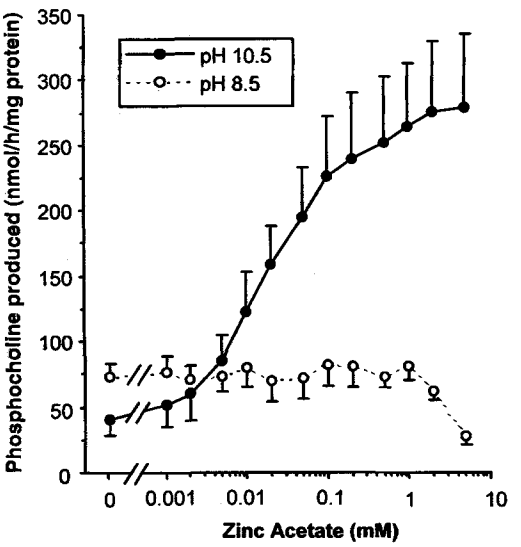


FIG. 2. Effect of zinc acetate concentration upon the phosphocholine-producing glycerophosphocholine phosphodiesterase activity of autopsied human brain. Activity was measured under a range of zinc acetate concentrations at pH 8.5 or 10.5 as indicated using 5 mM [^{3}H]glycerophosphocholine as substrate. The tissue preparations used in this experiment were homogenized in 50 mM HEPES/sodium hydroxide, pH 7.4 free of EDTA and EGTA. No chelating agents were added to the incubation buffer. Data represent the mean activity of three human temporal cortices analyzed in independent experiments; bars show the SEM.

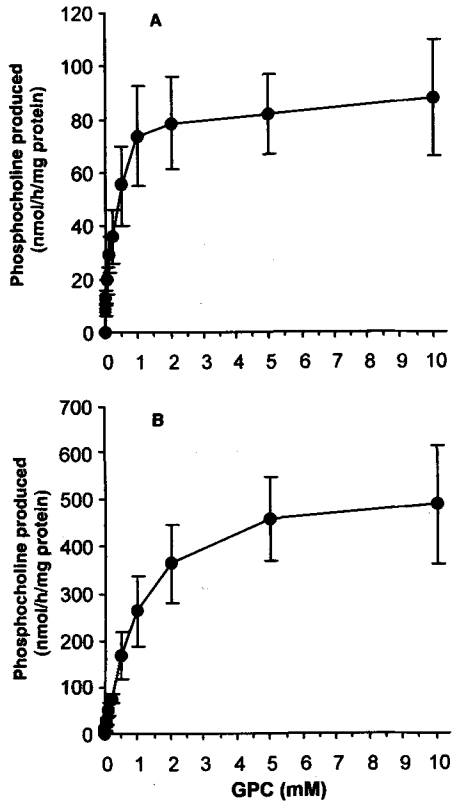


FIG. 3. Effect of substrate concentration upon the phosphocholine-producing GPC PD activity of autopsied human brain. GPC PD activity was measured in the presence of (A) 1 mM EDTA at pH 9.0, or (B) 5 mM zinc acetate at pH 10.5. The concentration of chelating agents contributed by the homogenization buffer were 12.5 μM EDTA and 12.5 μM EGTA. Data represent the mean activity of three human temporal cortices analyzed in independent experiments; bars show the SEM. See Figure 1 for abbreviation.

When assayed at pH 10.5, the enzyme was strongly activated in the presence of 5 mM cobalt chloride (1150 ± 108% of no additions), compared to 1400 ± 99% for zinc acetate (n = 3). However, 5 mM calcium chloride, sodium chloride, sodium acetate, or potassium chloride failed to activate the enzyme (data not shown), and 5 mM magnesium chloride caused only marginal activation (174 ± 27% of no additions; n = 3). In contrast, when assayed at pH 9.0, the rate of phosphocholine release was not altered by any of the aforementioned salts (data not shown).

The possibility that two phosphocholine-producing GPC PD activities were present in the preparation was investigated by pre-incubating the tissue homogenates with several compounds capable of covalently modifying amino acids (Table 1). Interestingly, acetic anhydride inhibited GPC PD activity by different degrees depending on whether GPC PD activity was assayed at pH 9.0 in the presence of EDTA (approximately 80% inhibition), or at pH 10.5 in the presence of 5 mM zinc acetate (approximately 25% inhibition). In contrast, when assayed at either pH 9.0 with 1 mM EDTA, or at pH 10.5 with 5 mM zinc acetate, the rate of phosphocholine release was unaffected by pre-incubation of tissue homogenates with bromophenacylbromide, *N*-ethylmaleimide,

TABLE 1
Effect of Amino-Modifying Compounds upon Phosphocholine-Producing GPC PD Activity in Autopsied Human Brain[a]

	Assay incubation conditions		
	0.5 mM GPC 1 mM EDTA pH 9.0	0.5 mM GPC 5 mM zinc acetate pH 10.5	5 mM GPC 5 mM zinc acetate pH 10.5
Bromophenacylbromide	112 ± 8	105 ± 2	113 ± 6
Diethylpyrocarbonate	33 ± 2	36 ± 2	45 ± 5
Phenylmethylsulphonylfluoride	116 ± 42	101 ± 3	94 ± 10
N-ethyl maleimide	105 ± 4	100 ± 5	107 ± 13
Acetic anhydride	22 ± 1	70 ± 1	75 ± 2

[a]Aliquots of temporal cortical homogenates were incubated at 37°C for 15 min in the presence of 10 mM modifier [dissolved in dimethylsulfoxide (DMSO); final DMSO concentration 4%]. The homogenates were then assayed for glycerophosphocholine phosphodiesterase (GPC PD) activity under the indicated reaction conditions. The concentration of modifier during the assay of GPC PD activity was 1 mM, due to the homogenates being diluted by assay buffer. The assay buffer also included 1 mM levamisole, 12.5 μM EDTA, and 12.5 μM EGTA contributed by the homogenization buffer. Results are expressed as mean percentage of activity in the presence of vehicle ± SEM for three brains analyzed in independent experiments. Control activity (i.e., pre-incubation in the presence of 4% DMSO) was 45 ± 14 for 0.5 mM GPC/1 mM EDTA/pH 9.0, 138 ± 38 nmol phosphocholine produced/h/mg protein for 0.5 mM GPC/5 mM zinc/pH 10.5, and 325 ± 39 nmol phosphocholine produced/h/mg protein for 5 mM GPC/5 mM zinc/pH 10.5.

and phenylmethylsulphonylfluoride (all at 10 mM concentration), while diethylpyrocarbonate inhibited both by approximately 65% (Table 1). The addition of 0.5 mM choline, phosphocholine, acetyl choline, 1-palmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, or arachidonic acid to the assay buffer did not significantly affect GPC PD activity under either assay condition (data not shown).

When GPC was substituted by 0.5 mM ^{14}C-GPE, no release of phosphoethanolamine or ethanolamine could be detected at either pH 9.0 in the presence of 1 mM EDTA, or at pH 10.5 in the presence of zinc acetate. In addition, the rate of GPC hydrolysis was not altered by the inclusion of 0.5 mM cold GPE, glycerophoshoserine, and glycerol-3-phosphate (data not shown).

Choline-producing enzymes. When homogenates of autopsied human brain were assayed for GPC PD activity at pH 10.5 in the absence of levamisole (an alkaline phosphatase inhibitor) with 0.5 mM GPC as substrate, choline was formed at a rate of 18.9 ± 7.3 nmol/h/mg protein (data not shown). The inclusion of 1 mM levamisole reduced choline formation to negligible levels, while the rate of phosphocholine release was unaffected (data not shown). This indicated that the released choline was derived from the hydrolysis of phosphocholine by alkaline phosphatase, and was not due to the actions of a choline-forming GPC PD activity. However, even in the presence of levamisole, other experiments, using biopsied rather than autopsied tissue, did suggest the presence of a distinct choline-producing activity. Due to the limited availability of biopsied tissue, only a preliminary investigation of this enzyme could be carried out. The choline-producing GPC PD activity in biopsied tissue was most active at pH 7.0 compared to pH 6.0, 8.0, 9.0, or 10.0 (data not shown), and required the presence of either magnesium or calcium ions for activity (Table 2). Furthermore, the addition of 0.5 mM GPE (i.e., equimolar to GPC) to the assay inhibited choline formation by 45 ± 12% (n = 3). Interestingly, an activity hydrolyzing GPE to form ethanolamine also was detected in biopsied tissue. The ionic and pH sensitivities of GPE hydrolysis were the same as that for GPC hydrolysis, and proceeded at a slightly lower rate (Table 2).

Glycerophosphodiesterase activities in autopsied compared to biopsied human brain. The stability of each enzyme post mortem was investigated by comparing the activity in autopsied temporal cortex with that in temporal cortical biopsies (Table 3). When assayed at pH 9.0 in the presence of 1 mM EDTA, phosphocholine-producing GPC PD activity was approximately half as active in autopsied tissue as it was in biopsied, while at pH 10.5 plus 5 mM zinc acetate activity was similar in both autopsied and biopsied tissues. However, significant hydrolysis of either GPC or GPE to form the free base was only detected in biopsied brain (Table 3).

TABLE 2
Choline/Ethananolamine-Producing Glycerophosphodiesterase Activity in Biopsied Human Brain[a]

	Substrate	
Addition	GPC (nmol/h/mg protein)	GPE (nmol/h/mg protein)
1 mM EDTA	0.6 ± 0.1	<0.5
5 mM calcium chloride	6.4 ± 1.2	4.0 ± 2.5
5 mM magnesium chloride	11.9 ± 2.9	8.7 ± 2.6

[a]Homogenates of three biopsied human temporal cortices were assayed for glycerophosphodiesterase in the presence of 0.5 mM glycerophosphodiester, 100 mM HEPES/sodium hydroxide, pH 7.0, and the indicated addition. The assay buffer also contained 1 mM levamisole, 25 μM EDTA, and 25 μM EGTA contributed by the homogenization buffer. Activities are given in nmol choline or ethanolamine produced/h/mg protein and are the mean ± SEM of three brains analyzed in independent experiments. GPE, glycerophosphoethanolamine. See Table 1 for other abbreviation.

TABLE 3
Glycerophosphodiesterase Activity in Autopsied and Biopsied Human Brain[a]

Incubation conditions	Autopsied tissue		Biopsied tissue	
	Phosphobase	Base	Phosphobase	Base
5 mM GPC/5 mM zinc acetate/pH 10.5	528 ± 101	<2.0	641 ± 58	<2.0
0.5 mM GPC/5 mM zinc acetate/pH 10.5	231 ± 79	<2.0	209 ± 25	<2.0
0.5 mM GPC/1 mM EDTA/pH 9.0	76 ± 12	<2.0	162 ± 33	<2.0
0.5 mM GPC/5 mM magnesium chloride/pH 7.0	16.2 ± 11.3	<2.0	20.3 ± 6.4	12.2 ± 0.9
0.5 mM GPE/5 mM magnesium chloride/pH 7.0	<2.0	<2.0	<2.0	9.2 ± 0.7

[a]Homogenates of four autopsied, or three biopsied, human temporal cortices were assayed for glycerophosphodiesterase activity under the indicated reaction conditions. The assay buffer also included 1 mM levamisole, 12.5 μM EDTA, and 12.5 μM EGTA contributed by the homogenization buffer. Activities are given in nmol product/h/mg protein and are given as mean ± SEM. See Table 1 for abbreviation.

DISCUSSION

GPC is an abundant breakdown product of choline phosphoglyceride. GPC is hydrolyzed by GPC PD to yield products which can be used in the resynthesis of choline phosphoglyceride. As such, GPC levels are frequently quantified as a measure of membrane turnover (1–6). Previous studies of GPC PD have identified several subforms of the enzyme in animal brain (7–9). However, surprisingly little is known about the characteristics of the human brain enzyme.

Our data suggest that human brain contains at least two enzymes capable of hydrolyzing GPC, one which forms phosphocholine as a product, the other producing free choline. In contrast to the GPC PD activity of rat brain (7), the most active enzyme in human brain was that which yielded phosphocholine, rather than choline, in agreement with that observed previously by others (1). The presence or absence of zinc ions in the assay incubation medium resulted in different characteristics being observed for the enzyme in terms of its substrate affinity, sensitivity to inhibition by acetic anhydride, pH optimum, and post mortem stability. Most strikingly, in the absence of zinc ions, the enzyme possessed a pH optimum of 9.0, which shifted to 10.5 with the addition of zinc acetate (see Fig. 1). A possible explanation for these observations is that zinc ions switch the conformation of a single enzyme between two states, one having a more alkaline pH optimum than the other. However, in such a scenario, as the amount of enzyme possessing a pH optimum of 10.5 increases, the proportion of the other form must decrease in a reciprocal manner. Thus, since stimulation of activity at pH 10.5 is not accompanied by a corresponding decrease at pH 9.0 (Fig. 1), or at pH 8.5 (Fig. 2; inhibition is only observed at high zinc ion concentrations), the possibility has to be considered that two phosphocholine-producing GPC PD activities are present in human brain, a zinc-sensitive, and a zinc-insensitive form. Zinc-sensitive GPC PD (Zn-GPC PD) activities have been described in both rat and bovine brain, and in *Bacillus cereus*, all of which display the highly alkaline pH optimum of the human enzyme assayed in the presence of zinc ions (7,18,19). Previous studies of this enzyme have indicated that it is localized predominantly to myelin (7,11,19). However, the tissue used in the present investigation was largely free of white matter. This suggests a more widespread localization, and consequently more general role, for the enzyme, although the possibility that the small myelin content of grey matter contributes all of the activity detected cannot be excluded. Since, in the presence of zinc, GPC PD possesses an unusually alkaline pH optimum, it is unclear how active the enzyme is *in vivo*. However, the availability of zinc is unlikely to be a limiting factor, since zinc concentrations in human brain [reported to be between 30 and 90 μg/g dry weight which, assuming a 90% water content of brain tissue, correspond to molar concentrations of between 50 and 140 μM (20)] are well above the EC_{50} of zinc for stimulation of the enzyme. Our results also suggest the presence of a zinc-insensitive phosphocholine-producing GPC PD activity in human brain which is active in the presence of EDTA. Similar, though not identical, enzymes have been described by others. Thus, rat brain also contains an EDTA-insensitive GPC PD activity, although it differs from that in human brain in terms of both its pH optimum and requirement of calcium ions for maximal activity (21). Moreover, Kanfer and McCartney (7) reported that rat brain microsomes contained a second phosphocholine-producing GPC PD activity which differed in its affinity for GPC from that of Zn-GPC PD, although no information was obtained regarding its metal ion sensitivity. If two phosphocholine-producing enzymes are present in human brain, it is unclear as to the roles and relative importance of each enzyme in brain. It has been suggested that Zn-GPC PD is the most physiologically relevant form of the enzyme regarding the choline phosphoglyceride degradative pathway, although little direct evidence exists to support this notion (18). Neither enzyme would appear to be involved in the metabolism of GPE, since we could not detect the production of phosphoethanolamine when GPE was used as a substrate. Similarly, the addition of GPE to the GPC PD assay did not inhibit the release of phosphocholine. The failure of Zn-GPC PD to hydrolyze GPE has previously been observed in rat brain preparations (7).

To assess the stability of phosphocholine-producing GPC PD activity post mortem, we compared their activities in autopsied brain to that in biopsied tissue. When assayed at pH 10.5 in the presence of zinc ions, GPC PD activity was similar in both tissue groups, suggesting that this enzyme is stable

post mortem. However, when assayed in the absence of zinc ions, GPC PD activity in autopsied temporal cortex was approximately half that in biopsied tissue, further suggesting the presence of two phosphocholine-producing enzymes, each one possessing a different post mortem stability.

A third GPC PD activity was detected in human brain which yielded the free base, rather than the phosphobase, as a product. This enzyme, which required divalent cations for activity and possessed a neutral pH optimum, could only be detected in biopsied tissue, thereby precluding future studies of this enzyme in disease conditions which routinely utilize autopsied brain. The phosphoester bond being cleaved, as well as the neutral pH optimum of this enzyme, could suggest that the reaction is catalyzed by a phospholipase D like activity (22,23). However, this possibility seems less likely given that the phospholipase D activities of rat and canine brain are either insensitive to, or inhibited by, divalent cations (23,24), in contrast to human brain choline-producing GPC PD, which is dependent upon divalent cations for activity. No evidence was found for a human brain homologue of the alkaline pH optima, calcium-dependent GPC PD activity identified in rat brain (8). Although the activity of the choline-producing enzyme, under optimal assay conditions, was much lower than that of the phosphocholine-producing enzymes, the observation that GPE inhibits the release of choline from labelled GPC, and that the hydrolysis of GPE to ethanolamine possesses similar characteristics to GPC hydrolysis, suggests that the same enzyme metabolizes both GPC and GPE. Thus, among the activities identified in human brain, the choline/ethanolamine-producing enzyme is unique in its ability to hydrolyze GPE, and, to our knowledge, represents the first identification of such an enzyme in brain. Since the other activity(s) identified in human brain did not catalyze detectable hydrolysis of GPE, the levels of brain GPE may be highly dependent upon the activity of this enzyme. In addition, while the role that the choline/ethanolamine-producing enzyme plays in the metabolism of GPC is presently unclear, the observation that this enzyme is optimally active at near-physiological pH, compared to the alkaline pH optimum of the phosphocholine-producing enzyme, suggests that the choline/ethanolamine-producing activity may represent a significant route of GPC hydrolysis *in vivo*. Finally, while a GPE-hydrolyzing activity has previously been identified in rat kidney, this enzyme differed from that in human brain in terms of its cation requirements and pH optimum (25).

Our failure to find a choline-producing GPC PD activity in autopsied human brain is in agreement with that of Blusztajn and colleagues (1), but is in contrast to the findings of Kanfer and colleagues (10), in which choline formation is described. However, these authors did not attempt to exclude the possibility that choline was being formed as a secondary product *via* the breakdown of phosphocholine by alkaline phosphatase. It may, therefore, be prudent to include such an inhibitor in future studies of this enzyme.

Previously, GPC PD activity has been measured in Alzheimer's diseased brains, and in multiple sclerosis plaques (1,10,11). It is now apparent that in these investigations the enzyme was assayed under conditions in which the majority of the GPC PD activity is due to a metal ion-independent GPC PD activity. Since our results suggest the presence of a second, zinc-sensitive enzyme, it may be valuable to reassess the status of GPC PD in these disorders.

ACKNOWLEDGMENTS

This work was supported by US NIH NINDS grant NS26034 (SJK) and the Ontario Mental Health Foundation (BMR).

REFERENCES

1. Blusztajn, J.K., Gonzalez-Coviella, I.G., Logue, M., Growdon, J.H., and Wurtman, R.J. (1990) Levels of Phospholipid Catabolic Intermediates, Glycerophosphocholine and Glycerophosphoethanolamine, Are Elevated in Brains of Alzheimer's Disease but Not of Down's Syndrome Patients, *Brain Res. 536*, 240–244.
2. Pettegrew, J.W., Keshavan, M.S., and Minshew, N.J. (1993) ^{31}P Nuclear Magnetic Resonance Spectroscopy: Neurodevelopment and Schizophrenia, *Schizophrenia Bulletin 19*, 35–53.
3. Minshew, N.J., Goldstein, G., Dombrowski, S.M., Panchalingam, K., and Pettegrew, J.W. (1993) A Preliminary ^{31}P MRS Study of Autism: Evidence for Undersynthesis and Increased Degradation of Brain Membranes, *Biol. Psychiatry 33*, 762–773.
4. Smith, C.D., Gallenstein, L.G., Layton, W.J., Kryscio, R.J., and Markesbery, W.R. (1993) ^{31}P Magnetic Resonance Spectroscopy in Alzheimer's and Pick's Disease, *Neurobiol. Aging 14*, 85–92.
5. Nitsch, R.M., Blusztajn, J.K., Pittas, A.G., Slack, B.E., Growdon, J.H., and Wurtman, R.J. (1992) Evidence for a Membrane Defect in Alzheimer's Disease Brain, *Proc. Natl. Acad. Sci. USA 89*, 1671–1675.
6. Nitsch, R.M., Blusztajn, J.K., Doyle, F.M., Robitaille, Y., Wurtman, R.J., Growdon, J.H., and Kish, S.J. (1993) Phospholipid Metabolite Levels Are Altered in Cerebral Cortex of Patients with Dominantly Inherited Olivopontocerebellar Atrophy, *Neurosci. Lett. 161*, 191–194.
7. Kanfer, J.N., and McCartney, D.G. (1989) Glycerophosphocholine Phosphocholine Phosphodiesterase Activity of Rat Brain Myelin, *J. Neurosci. Res. 24*, 231–240.
8. Spanner, S., and Ansell, G.B. (1982) Activation of Glycerophosphocholine Phosphodiesterase in Rat Forebrain by Ca^{2+}, *Biochem. J. 208*, 84–85.
9. Dawson, R.M.C. (1956) Liver Glycerylphosphorylcholine Diesterase, *Biochem. J. 62*, 689–693.
10. Kanfer, J.N., Pettegrew, J.W., Moossy, J., and McCartney, D.G. (1993) Alterations of Selected Enzymes of Phospholipid Metabolism in Alzheimer's Disease Brain Tissue as Compared to Non-Alzheimer's Demented Controls, *Neurochem. Res. 18*, 331–334.
11. Janzen, L., Tourtellotte, W.W., and Kanfer, J.N. (1990) Glycerylphosphocholine Phosphocholine Phosphodiesterase Activity Is Reduced in Multiple Sclerosis Plaques, *Exper. Neurol. 109*, 243–246.
12. Parnetti, L., Abate, G., Bartorelli, L., Cucinotta, D., Cuzzupoli, M., Maggioni, M., Villardita, C., and Senin, U. (1993) Multicentre Study of L-α-glyceryl-phosphorylcholine vs. ST200 among Patients with Probable Senile Dementia of Alzheimer's Type, *Drugs and Aging 3*, 159–164.
13. Bronzetti, E., Felici, L., and Amenta, F. (1994) Effect of Ipsilateral Lesioning of the Nucleus Basalis Magnocellularis and of L-α-glyceryl Phosphorylcholine Treatment on Choline Acetyl-

transferase and Acetylcholinesterase in the Rat Fronto-Parietal Cortex, *Neurosci. Lett. 164*, 47–50.
14. Spanner, S., and Ansell, G.B. (1987) The Hydrolysis of Glycerophosphocholine by Rat Brain Microsomes: Activation and Inhibition, *Neurochem. Res. 12*, 203–206.
15. Bartlett, G.R. (1959) Phosphorous Assay in Column Chromatography, *J. Biol. Chem. 234*, 466–468.
16. Petty, A.C., and Scrutton, M.C. (1993) Release of Choline Metabolites from Human Platelets: Evidence for Activation of Phospholipases D and of Phosphatidylcholine-Specific Phospholipase C, *Platelets 4*, 23–29.
17. Kanoh, H., Kanaho, Y., and Nozawa, Y. (1991) Activation and Solubilization by Triton X-100 of Membrane-Bound Phospholipase D of Rat Brain, *Lipids 26*, 426–430.
18. Sok, D.-E., and Kim, M.R. (1992) Characterization of a Zn^{2+}-Requiring Glycerophosphocholine Cholinephosphodiesterase Possessing *p*-Nitrophenylphosphocholine Phosphodiesterase Activity, *Biochem. J. 286*, 435–440.
19. Yuan, J., and Kanfer, J.N. (1994) Purification and Properties of a Glycerophosphocholine Phosphodiesterase from Bovine Brain Myelin, *Neurochem. Res. 19*, 43–48.
20. Pfeiffer, C.C., and Braverman, E.R. (1982) Zinc, the Brain and Behavior, *Biol. Psychiatry 17*, 513–532.
21. Abra, R.M., and Quinn, P.J. (1976) Some Characteristics of *sn*-Glycero-3-Phosphocholine Diesterases from Rat Brain, *Biochim. Biophys. Acta 431*, 631–639.
22. Ghosh, S.S., and Franson, R.C. (1992) Use of [1-^{14}C]Oleate Labelled Autoclaved *Escherichia coli* as a Membranous Substrate for Measurement of *in vitro* Phospholipase D Activity, *Biochem. Cell Biol. 70*, 43–48.
23. Nishida, A., Shimizu, M., Kanaho, Y., Nozawa, Y., and Yamawaki, S. (1992) Characterization of Phospholipase D in a Cell-Free System of Cultured Cells Derived from Rat Frontal Cortex, *Brain Res. 595*, 12–16.
24. Chattopadhyay, J., Natarajan, V., and Schmid, H.H.O. (1991) Membrane-Associated Phospholipase D Activity in Rat Sciatic Nerve, *J. Neurochem. 57*, 1429–1436.
25. Baldwin, J.J., and Cornatzer, W.E. (1968) Rat Kidney Glycerylphosphorylcholine Diesterase, *Biochim. Biophys. Acta 164*, 195–204.

[Received April 3, 1995, and in final revised form July 6, 1995; Revision accepted October 12, 1995]

Regulation of Cholesterol Synthesis in Four Colonic Adenocarcinoma Cell Lines

Sonia R. Cerda, John Wilkinson IV, and Selwyn A. Broitman*

Boston University School of Medicine, Department of Microbiology and The Mallory Institute of Pathology, Boston City Hospital, Boston, Massachusetts 02118

ABSTRACT: Colon tumor cells, unlike normal human fibroblasts, exhibited an uncoupling of low density lipoprotein (LDL)-derived cholesterol from cellular growth, when endogenous cholesterol synthesis was inhibited by mevinolin, a hydroxymethylglutaryl–CoA reductase (HMG–CoAR) competitive inhibitor [Fabricant, M., and Broitman, S.A. (1990) *Cancer Res. 50,* 632–636]. Further evaluation of cholesterol metabolism was conducted in two undifferentiated (SW480, SW1417) and two differentiated (HT29, CACO2) colonic adenocarcinoma (adeno-CA) cell lines and an untransformed human fibroblast, AG1519A. Cells grown in monolayer culture to near subconfluency were used to assess endogenous cholesterol synthesis by ^{14}C-acetate incorporation, in response to the following treatments in lipoprotein-deficient serum (LPDS)-supplemented minimum essential medium (MEM): LPDS alone, LDL, mevinolin, mevinolin with LDL, and 25-hydroxy-cholesterol (25-OH-CH). Complete fetal bovine serum (FBS)-supplemented MEM was used as control. All colon tumor lines exhibited similarly high endogenous cholesterol synthesis in both FBS and LPDS relative to the fibroblasts which demonstrated low basal levels in FBS and maximal synthesis in LPDS. LDL treatment did not inhibit cholesterol synthesis in colon tumor cells, but suppressed that in the fibroblast by 70%. Sterol repression of cholesterol synthesis mediated by 25-OH-CH occurred in all cells. Mevinolin caused a reduction in cholesterol synthesis in the colonic cancer cell lines, which was not further decreased by concurrent addition of LDL. In contrast, in mevinolin-treated fibroblasts, LDL further inhibited cholesterol synthesis. When the effect of cell density on cholesterol synthesis regulation was evaluated under conditions of sparse density in SW480 and SW1417, results indicated that (i) basal rates of cholesterol synthesis were higher, (ii) LDL inhibited cholesterol synthesis more effectively, and (iii) mevinolin or 25-OH-CH had a more pronounced effect than in subconfluent cells. Evaluation of LDL receptor activity through ^{125}I-LDL binding and internalization studies demonstrated LDL receptor expression was reduced by 37% in normal density cells relative to the low density cultures. In contrast to cholesterol synthesis, exogenous LDL could inhibit LDL receptor activity at both densities. Thus subconfluent growing colonic adenoCA cell lines retain the capacity for sterol repression, but, in contrast to normal fibroblasts, exhibit a high endogenous cholesterol synthesis which LDL cannot regulate. *Lipids 30,* 1083–1092 (1995).

Cholesterol is required for growth of cells (1). This sterol may be obtained either by *de novo* synthesis from acetate, which is catalyzed by the enzyme 3-hydroxymethylglutaryl–CoA reductase (HMG–CoAR), or *via* uptake of exogenous cholesterol from the environment, primarily as low density lipoprotein (LDL) (2). A relationship between the neoplastic state and loss of feedback inhibition in cholesterol metabolism was first suggested by Siperstein *et al.* (3), who described a failure of dietary cholesterol to regulate *de novo* synthesis in hepatoma and prehepatoma cells. Rapidly proliferating cancer cells may have a higher cholesterol requirement for membrane or synthetic purposes; consequently, these increased demands for cholesterol can be met either by increasing endogenous synthesis (4,5) or uptake (6–13) of exogenous cholesterol as LDL, or both (14).

The rate-limiting step in the cholesterol biosynthetic pathway is catalyzed by HMG–CoAR (15). Increased activity of this enzyme increases its direct product, mevalonate, precursor to sterols and nonsterol isoprenoids, such as ubiquinone, dolichol, and isopentenyl tRNA, which are all necessary for cell growth (16). Mevalonate also incorporates into isoprenylated proteins (17), including such signal transduction participants as ras and other GTP binding proteins (18). An increase in the expression of HMG–CoAR has been described in certain tumors relative to normal tissue (19–21), indicating its importance in cellular growth control.

Cells grown in culture exhibit a preference for cholesterol *via* receptor-mediated uptake of LDL (22). Inhibition of cholesterol synthesis by compactin, a fungal metabolite known to competitively inhibit HMG–CoAR (23), renders growth of fibroblasts, Chinese hamster ovary, and raji lymphoma cells dependent on the LDL receptor (LDLR) (24). When the four lines used in these experiments were grown in lipoprotein-deficient serum (LPDS)-supplemented media in the presence of mevinolin (at a dose which inhibits growth by 50%), LDL was unable to reverse growth inhibition, in direct contrast to the human fibroblast, where growth was totally restored (25).

*To whom correspondence should be addressed at Boston University School of Medicine, Department of Microbiology, 15 Stoughton St., Boston, MA 02118.

Abbreviations: ACAT, acyl cholesterol acyl transferase; adeno-CA, adenocarcinoma; ANOVA, analysis of variance; BSA, bovine serum albumin; FBS, fetal bovine serum; HMG–CoAR, 3-hydroxy-3-methylglutaryl–CoA reductase; 25-OH-CH, 25-hydroxy-cholesterol; LDL, low density lipoprotein (quantities refer to measured or derived protein); LDLR, LDL receptor; LPDS, lipoprotein-deficient serum; MEM, minimum essential medium; PBS, phosphate-buffered saline; TBS, tris-buffered.

Copyright © 1995 by AOCS Press

It has been demonstrated that small intestinal cells do not use exogenous LDL for growth when endogenous cholesterol synthesis is inhibited by mevinolin, though LDL receptors may be present and this lipoprotein internalized by the cells (26–28). However, discrepancies still exist as to whether or not intestinal cells preferentially use endogenous cholesterol for membrane synthesis (29). In order to further elucidate previous findings, we studied cholesterol metabolism in four colonic adenocarcinoma (adenoCA) cell lines, two of which (HT29, CACO2) differentiate *in vitro* under appropriate conditions to small intestinal cells (30,31). Results suggest a lack of regulation upon endogenous cholesterol synthesis by LDL in subconfluent growing colonic adenoCA cells, similar to the metabolism of a differentiated small intestinal cell phenotype.

MATERIALS AND METHODS

Cell culture. Four human colonic cancer cell lines, SW480, SW1417, HT29, and CACO2, were obtained from the American Type Culture Collection (Rockville, MD). Stock cultures were grown in monolayer in plastic 75 cm^2 flasks and incubated at 37°C in a 5% CO^2/95% air atmosphere. Culture medium consisted of Eagle's minimal essential medium (MEM) containing antibiotics (penicillin, 200 units/mL; streptomycin, 0.2 mg/mL), glutamine (0.2 mg/mL), and nonessential amino acids and nonessential vitamins both at 10 mL of 100X solution (GIBCO, Grand Island, NY), per 500 mL MEM and supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). Cells were plated at a density of 10^6 cells per flask, and cultures divided weekly (1:19 or 1:20) using 0.05% trypsin/0.02% disodium EDTA. After removal of media, cells were rinsed with isotonic phosphate-buffered saline (PBS) (pH 7.4), and 4 mL of trypsin added. Flasks were incubated at 37°C until cells began to detach, after which 4–6 mL of fresh medium was added. Cells used in experiments are grown in plastic 35 mm^2 dishes. Normal human skin fibroblast cells (AG1519A), obtained from the Institute for Medical Research (Camden, NJ), were grown as monolayers in MEM supplemented with antibiotics, glutamine, nonessential amino acids, and 20% FBS. Stock cultures were plated at a density of 1×10^6 per 75 cm^2 flask and divided weekly (1:4) using trypsin/EDTA. Cell cultures were assayed for the presence of mycoplasma using the Gen-Prove Mycoplasma T.C. 11 Rapid Detection System (San Diego, CA).

Preparation of LPDS. Lipoproteins were removed from FBS by density gradient centrifugation (32). FBS was adjusted to a density of 1.21 g/mL with solid potassium bromide (64.4 g KBr per 200 mL FBS) and centrifuged for 48 h at 5°C at 244,000 $\times$ g_{max} (45,000 rpm in a 50.2ti rotor in a Beckman L8-70 ultracentrifuge; Beckman Instruments, Fullerton, CA). The top lipoprotein fraction (d < 1.21 g/mL) was removed, and the bottom lipoprotein-deficient fraction dialyzed extensively against 0.01% EDTA/double-distilled water (pH 8) and then PBS. The LPDS was sterilized by passage through a 0.22 μ filter.

Preparation of mevinolin. This reagent was kindly provided by A. Alberts of Merck Sharp & Dohme (Rahway, NJ) in the lactone form. Saponification was carried out by adding 0.1 mL absolute ethanol and 0.1 mL of 0.1 N NaOH to 4 mg of mevinolin and by heating at 50°C for two hours. Mevinolin was neutralized with 5% HCl to pH 7.3. Stock solutions (4 mg/mL) were prepared by bringing the volume to 1 mL with dimethyl sulfoxide and stored at –20°C. At the concentrations used, dimethyl sulfoxide was found to have no effect on cell growth. Mevinolin was diluted in MEM to the concentrations used in experiments. All the studies regarding the use of mevinolin were conducted at a 1 μM concentration as published by this laboratory (25).

Protein determinations. Proteins were determined by the bicinchoninic acid reagent method (Pierce Chemical Company, Rockford, IL). A standard curve was prepared using a stock bovine serum albumin standard in sample diluent. Samples and standard are mixed with the protein reagent and absorbance read at 562 nm by spectrophotometry.

Cholesterol synthesis assay. Cells were seeded in six-well (35 mm^2) dishes at 20,000 cells/well in triplicate per group studied per experiment, in MEM supplemented media with 10% (SW480, SW1417, and HT29) or 20% (CACO2 and AG1519A) FBS. All cells were grown to a subconfluent to confluent growth, termed normal density (≈80% of the plate was covered after day 5), except where specified as "LD," meaning low density or sparse growth (≈30% was covered, after day 3). After day 5 (day 3 for LD cultures), cells were then rinsed with Dulbecco's PBS and switched to a LPDS based medium (1 mL/well). After 24 to 48 h, the media was replaced with media containing 5 μCi/mL of 2-^{14}C-acetate (51 mCi/mmole NEN), and the following treatments: (i) FBS (ii) LPDS, the basal media for all subsequent treatments (each of which contain 10% LPDS), (iii) LDL (50 μg protein/mL), (iv) 25-OH-CH (25-OH-CH at 0.5 μg/mL + cholesterol at 16 μg/mL), (v) mevinolin at a 1 μM concentration, and (vi) mevinolin + LDL. After 24-h incubation, the medium was removed and saved; monolayers were harvested in 1 mL 0.1 N NaOH and pooled with their respective saved media to be saponified following the methodology of Brown *et al.* (32). Briefly, these mixtures were treated with 0.5 mL 50% KOH, 3 mL ethanol, and (1,2) ^{3}H-cholesterol (10^5 cpm, 50 Ci/mmole) as internal standard. After saponification at 80°C for two hours, unsaponifiable lipids were extracted three times with petroleum ether followed by two washes with NaOH. Samples were dried with nitrogen gas, resuspended in chloroform, spotted on silica gel "G" thin-layer chromatography plates, and separated using chloroform as a running solvent to isolate the ^{14}C-cholesterol with appropriate markers. This methodology discriminated 27C sterols from lanosterol and squalene, which had higher R_f values. Although this running system does not resolve cholesterol from other 27C sterols, gas chromatography analysis of the derivatized lipids from the scrapings showed a predominance of cholesterol with some desmosterol and 7α-dehydrocholesterol (data not shown). Cholesterol synthesis was evaluated as nanomoles of

acetate incorporated into cholesterol per mg cellular protein, and these values normalized to the LPDS control within each experiment. Results of challenge with various treatments were expressed as percentage of LPDS control. Data was analyzed by general linear model analysis of variance (ANOVA) to determine significant differences between groups.

^{125}I-LDL binding to colonic adenoCA cell lines. The ^{125}I-LDL binding assays were conducted using the following methodology adapted from Goldstein *et al.* (33). Cells were seeded at 2.5 and 15×10^4 cells per well in three wells each of six-well plates to generate contrasting conditions of normal, and low density within individual assay plates. After two days' growth in FBS-supplemented MEM, cells were washed with PBS and changed to media supplemented with 10% LPDS, which was prepared in-house from FBS (Sigma) to generate maximal responses in terms of receptor activity. After 24 h, the cells received treatments in LPDS-based MEM for a duration of 24 h. Following treatments, the cells were incubated in 1 mL LPDS/MEM with 5–10 μg/mL ^{125}I-LDL (Biomedical Technologies Inc., Stoughton, MA) for five hours. Within an experiment, two wells of a six-well dish are used to measure hot or total binding per treatment at either density state. To control for nonspecific binding, one well received 25- to 40-fold increase of cold LDL (125–200 μg/mL) above the level of the hot LDL in the media. One empty, cell-free, six-well plate was treated with media, washed, treated, and incubated just as the other groups, and used as a blank.

Surface binding. After incubation for five hours, a steady state is reached in which binding, internalization, and degradation of the lipoprotein are equilibrated (33). The cells were placed on an ice bed, and rinsed 5 times with 2 mL ice-cold tris-buffered saline (TBS)-A buffer (50 mM Tris/150 mM NaCl/2 mg/mL albumin at pH 7.4), once with ice-cold TBS buffer (TBS-A with no albumin), and finally incubated with 1.5 mL of dextran sulfate release buffer (50 mM NaCl/10 mM HEPES/4 mg/mL dextran sulfate at pH 7.4) for one hour at 4°C. Polyanionic macromolecules such as dextran sulfate or heparin sulfate cause LDL to be released from its receptor; thus the activity of the released tracer is a measurement of the surface binding capacity for the cells. Following this incubation, 750 μL aliquots were taken for gamma counting.

Internalization. The major count found in this assay represents the LDL that is internalized by the cells, which has not yet been degraded. In fibroblasts this will typically be 80–90% of the total cell associated radioactivity (33). To measure internalization, the cells are returned to the ice bed and washed two times with 2 mL TBS buffer. Then, the monolayers are dissolved in 1.5 mL hot 60°C NaOH (0.1N), and aliquots are taken for gamma counting (750 μL) and protein (20 μL) measurement.

RESULTS

Colon tumor cells were evaluated for their ability to synthesize cholesterol in response to different sterol treatments. Cells were grown in culture to a subconfluent state to limit any growth-rate dependent effects of replication upon the results. At subconfluency, cells were deprived of exogenous sterols through culture for 24 h in LPDS-based MEM. Under these conditions, fibroblasts depend upon endogenous cholesterol synthesis for growth (2). Cells were then subjected to subsequent treatments. All data is normalized as a percentage of the individual experiments' LPDS control group.

FBS provides cells with a sterol-rich medium, generating a reference point to determine levels of endogenous synthesis under "normal" growth conditions. The colonic cell lines exhibited similarly high basal rates of cholesterol synthesis in both FBS- and LPDS-supplemented media. The fibroblast presented low basal levels of cholesterol synthesis when grown in FBS (Fig. 1A–E), suggesting an increased reliance upon endogenous synthesis in subconfluent colonic adenoCA cells.

LDL is the primary cholesterol source in man (2), and certain cells in culture preferentially derive this sterol from LDL in the serum of culture media, synthesizing cholesterol at a low rate (22). When colonic tumor cells grown in LPDS were treated with this lipoprotein, cholesterol synthesis was not suppressed (Fig. 1A–D), as occurs in the fibroblast (Fig. 1E), indicating an inability of this lipoprotein to regulate endogenous cholesterol synthesis.

Regulation by LDL of HMG–CoAR has been shown to occur both transcriptionally and post-transcriptionally *via* a putative oxysterol intermediate (16). In order to test if colonic tumor cells can demonstrate a functional sterol-mediated suppression of this enzyme, the ability of the oxysterol 25-OH-CH to repress cholesterol synthesis was tested. All cells were found to exhibit a decrease in cholesterol synthesis when treated with this oxysterol in LPDS (Fig. 1A–E), indicating that oxysterol regulatory mechanisms remained functional in these lines.

Most of the work in cholesterol metabolism in human fibroblasts has taken place at a confluent state (35–37) in order to avoid replication-dependent factors upon the results. However, alterations in confluency state can cause certain cultured colonic cell lines such as $CACO_2$ cells to differentiate and gain small bowel phenotypic characteristics. Therefore, we examined whether cell density had an effect on the ability of sterols to regulate cholesterol synthesis in two lines which are not known to differentiate in culture to a small bowel phenotype, the undifferentiated colonic adenocarcinomas SW480 and SW1417 (Fig. 2). The sterols in FBS and LDL were found to downregulate endogenous cholesterol synthesis in low density growing cells (≈30% of the dish is covered at three days), in direct contrast to subconfluent (≈80% of the dish was covered) cells. Additionally, the rates of *de novo* cholesterol synthesis (see Table 1) in cells cultured at lower densities were higher than at subconfluency, implying a greater demand for endogenously synthesized cholesterol in low density cells. However, while endogenous cholesterol synthesis was inhibited by LDL in normal fibroblasts at both low and normal densities of growth, colon tumor cells were

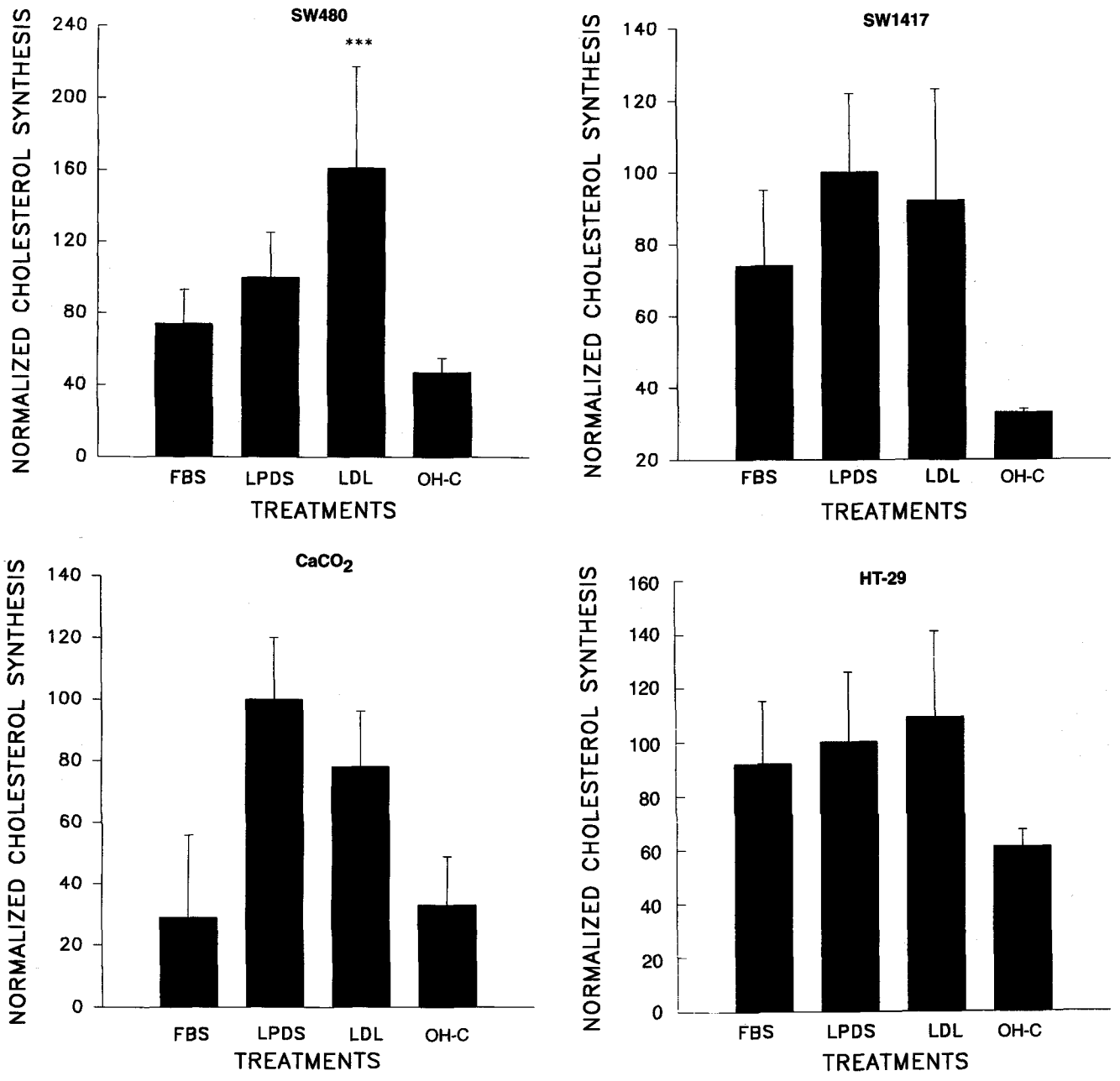
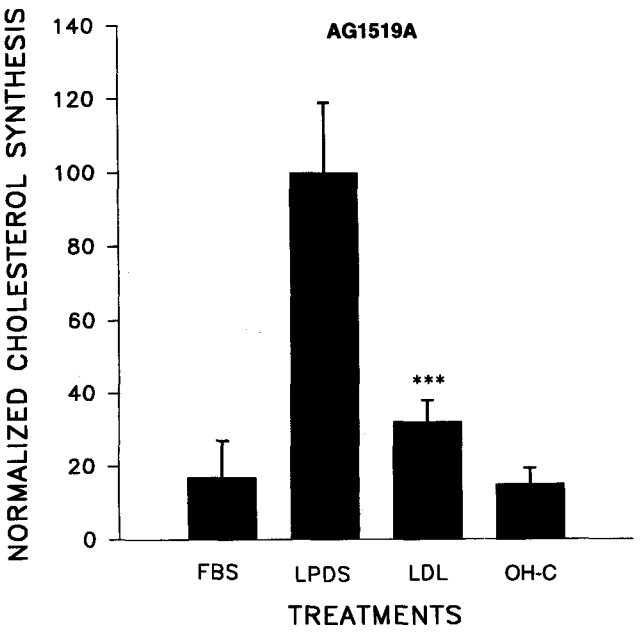


FIG. 1. A–E: (continued on next page).

only repressed by this sterol when growing at low density. It is of interest that control studies have shown the same effects at 1.5 h of treatment, even when the specific activity was diluted to one-third of the original values used in this study (data not shown).

LDLR activity was then analyzed in SW480 cells under these two density culture conditions. LDLR is reported as ng ^{125}I-LDL bound per mg cell protein, and the mean of three assays is depicted in Tables 2 and 3. The mean LDLR activity for these cells at both density states in LPDS-supplemented media is low (57 to 90 ng LDL/mg cell protein internalized count) relative to the fibroblast, which has a reported 100 and 600 ng/mg for surface and internalized activity, respectively (33). Thus, as previously speculated (25), this tumor cell line has a lowered LDLR activity relative to fibroblasts. This is consistent with an enhanced cholesterol synthesis rate in these cells, as strictly sterol-regulated genes, such as the receptor, would be repressed by such an increase in intracellular sterols. The LDLR activity between density states across all treatments was not statistically different by ANOVA (α 0.05). Cells were treated with LDL (5, 50, 300 μg/mL), or 25-OH-CH (3 μg/mL) in basal LPDS media to evaluate the effect of external sterols on LDLR activity. As seen in Table 2, addition of 5, 50, and 300 μg/mL LDL resulted in a diminution of LDLR activity at both density states. This difference was statistically significant at both densities between all treated groups and the LPDS controls within each density state (ANOVA $P <$ 0.01; Bonferoni control test for multiple comparisons). Addi-



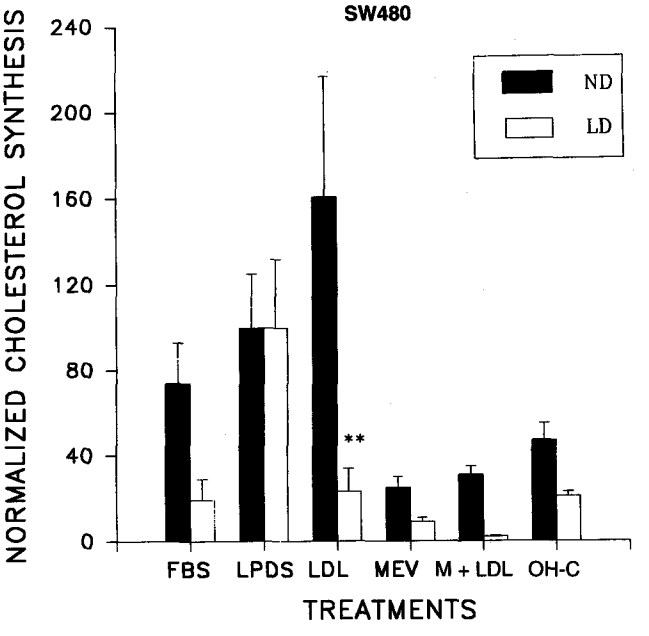


FIG. 1. A–E: Cellular cholesterol synthesis in four colonic adenocarcinoma cell lines ($CACO_2$, HT29, SW480, SW1417; American Type Culture Collection, Rockville, MD), and a normal human fibroblast AG1519A (Institute for Medical Research Camden, NJ). Cells were grown to subconfluency (80% of the plate was covered), incubated with ^{14}C-acetate, nonsaponifiable lipids were extracted and subjected to thin-layer chromatography as indicated in the Materials and Methods section. Nanomoles of acetate per mg cellular protein were determined per sample, and these values were normalized to the lipoprotein-deficient serum (LPDS) control within each experiment. Results of challenge with various treatments are expressed as percentage of LPDS control and depicted as bar graphs, where the filled area represents the mean of three different experiments carried out in triplicate. Open areas above the bars represent the standard deviation of the mean. Significance of findings was evaluated by general linear model analysis of variance comparing: low density lipoprotein (LDL) treatments with LPDS controls. FBS, Fetal bovine serum; OH-C, 25-hydroxy-cholesterol.

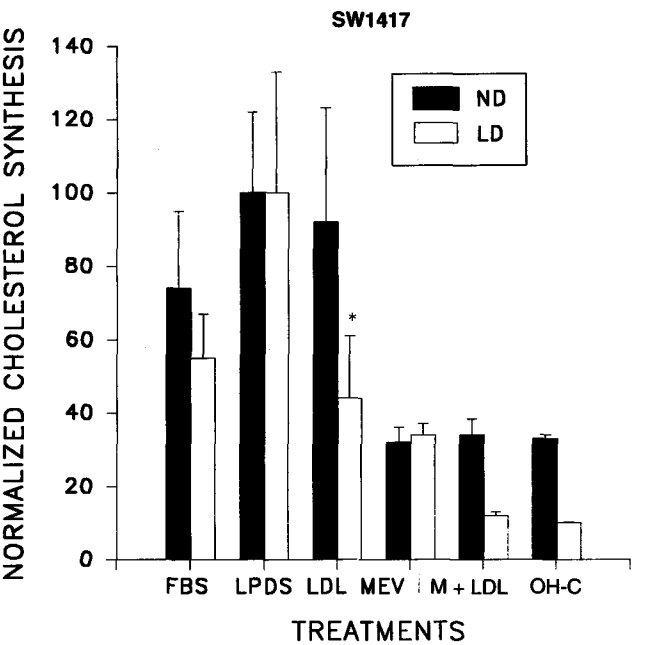


FIG. 2. A, B: Comparison of cellular cholesterol synthesis in two colonic adenocarcinoma cell lines between subconfluent and lower density culture conditions. SW480 and SW1417 cells were grown to a low density state (30% of the plate was covered), and treated as described for Figure 1. Results are expressed as indicated for Figure 1, except that filled bars represent subconfluent cultures [normal density (ND)] depicted in Figure 1 (C, D), and open bars represent cultures seeded and grown to sparser densities [low density (LD)]; Mev, mevinolin; M + LDL, mevinolin + LDL. See Figure 1 for other abbreviations and company sources.

tion of 25-OH-CH (3 μg/mL) caused significant ($P < 0.01$) inhibition of the LDLR activity vs. the LPDS control group.

In fibroblasts cultured in lipoprotein-deficient medium, inhibition of cholesterol synthesis with the HMG–CoAR inhibitor compactin renders their growth dependent upon LDL-derived cholesterol (24). To assess the effects of a similar HMG–CoAR inhibitor, mevinolin, upon cholesterol metabolism in colonic adenocarcinomas, we determined its impact upon cholesterol synthesis. In order to minimize differences in evaluating the effects of this inhibitor between the lines when analyzing results, the concentration of mevinolin remained the same for all the cells. As shown in Figure 3, mevinolin inhibited cholesterol synthesis in all lines.

This report is consistent with previous findings, where LDL did not reverse growth inhibition imposed by mevinolin (25). In the current study, when the four colonic adenoCA cell lines were grown in the presence of mevinolin, concurrent addition of LDL had no effect upon cholesterol synthesis in the tumor lines (Fig. 3). In contrast, LDL further inhibited cholesterol synthesis in the fibroblast. These effects also were evaluated in SW480 and SW1417 cells growing at low density (Fig. 2). These cells responded to simultaneous treatment with both mevinolin and LDL by further suppressing cholesterol synthesis, similar to the fibroblast. Additionally, cholesterol synthesis was inhibited to a greater extent by treatment

TABLE 1
Nanomoles of ^{14}C-Acetate Incorporated into Cholesterol per Milligram of Cellular Protein (in 24 h)[a]

	Treatment	SW1417	SW480	AG1519A
Low density	LPDS	13.5 ± 5.2	113.1 ± 43.6	17.1 ± 5.7
	LDL	4.2 ± 1.9	37.8 ± 6.4	4.8 ± 0.7
Normal density	LPDS	5.25 ± 2.2	7.35 ± 2.7	7.5 ± 1.9
	LDL	5.9 ± 1.5	12.15 ± 4.5	1.95 ± 0.45

[a]Cells were grown to a low density state (30% of the plate was covered), or 80% confluent and treated with lipoprotein-deficient serum (LPDS) or low density lipoprotein (LDL) 50 μg/mL (LDL) in the presence of ^{14}C-acetate. Nonsaponifiable lipids were extracted and subjected to thin-layer chromatography as indicated in the Materials and Methods section. Cholesterol synthesis is reported as nanomoles of acetate incorporated per mg cellular protein. Results are expressed as means ± SE of a representative of three experiments, n = 3. SW1417 and SW480 are colonic adenocarcinoma cell lines (American Type Culture Collection, Rockville, MD). AG1519A (Institute for Medical Research, Camden, NJ) is a normal human fibroblast.

TABLE 2
LDLR Activity in SW480 Cells Cultured at Two Different Density Conditions in Response to Various Treatments[a]

	Normal density (internal)		Normal density (surface)		Low density (internal)		Low density (surface)	
Treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LPDS	57.3 ± 5.9		14.1 ± 5.7		89.8 ± 40.1		20.0 ± 13.9	
LDL 5 μg	30.6 ± 8.1		9.0 ± 5.4		31.5 ± 23.6		5.7 ± 4.1	
LDL 50 μg	30.6 ± 8.1		4.8 ± 2.6		34.8 ± 20.3		7.3 ± 5.3	
LDL 300 μg	10.2 ± 4.1		0.6 ± 0.4		7.6 ± 6.8		0.0 ± 0.0	
25-OH-CH 3 μg	18.9 ± 8.3		6.6 ± 3.5		26.1 ± 18.4		10.5 ± 9.2	

[a]SW480 cells were cultured at normal and low density states, pretreated with LPDS-supplemented media for 24 h, then treated for 24 h with the LPDS-based media alone or supplemented with increasing concentrations of human LDL as indicated or with 25-hydroxy-cholesterol (25-OH-CH) (3 μg/mL). Cells were evaluated as described under methods for their ability to bind (surface activity) and internalize ^{125}I-LDL. Table 1 activity is reported as ng of radiolabeled LDL per mg cellular protein (mean and standard error of six measurements taken over three experiments). See Table 1 for company source and other abbreviations.

with mevinolin and 25-OH-CH as compared to normal density growing subconfluent cells (Fig. 2).

DISCUSSION

Stange and Dietschy (38) first demonstrated an independent regulation between LDL uptake and cholesterol synthesis in rat intestinal epithelium. This work and that of others (26–28,39) led to a proposed model for the compartmentalization of cholesterol metabolism and cellular growth in intestinal cells (40). The present study shows that colonic adenoCA cell lines exhibit different responses in cholesterol synthesis to treatment with: the sterols in FBS, LDL, mevinolin, and mevinolin + LDL. Our results demonstrate that in cultures supplemented with LDL at 50 μg/mL in LPDS-based medium, cholesterol synthesis was not found to be inhibited, as opposed to the fibroblast (Fig. 1). This unresponsiveness to LDL could reflect: (i) relative deficiency in LDLR expression or activity, (ii) an altered compartmentalization, trafficking, or storage of LDL-derived cholesterol into non-regulatory cellular pools, and (iii) a deregulation in the mechanisms by which sterols downregulate HMG–CoAR. The latter hypothesis was deemed unlikely because data indicated normal sterol regulatory function in these cells by 25-OH-CH mediated sterol repression (see Fig. 1A–E).

The inclusion of colonic tumor-derived cell lines into the

TABLE 3
LDLR Activity in SW480 Cells Cultured at Two Different Density Conditions in Response to Various Treatments[a]

	Normal density (internal)		Normal density (surface)		Low density (internal)		Low density (surface)	
Treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LPDS	100 ± 4.4		100 ± 14.2		100 ± 11.7		100 ± 3.2	
LDL 5 μg	50.2 ± 9.2		41.1 ± 18.9		33.9 ± 12.2		19.5 ± 12.3	
LDL 50 μg	51.9 ± 10.0		23.3 ± 9.6		26.3 ± 9.0		36.7 ± 5.0	
LDL 300 μg	20.1 ± 6.6		6.6 ± 3.6		19.5 ± 13.3		NA ± NA	
25-OH-CH 3 μg	29.4 ± 10.9		32.1 ± 12.3		14.2 ± 9.3		52.2 ± 21.3	

[a]SW480 cells were cultured at normal and low density states, pretreated with LPDS supplemented media for 24 h, then treated for 24 h with the LPDS-based media alone or supplemented with increasing concentrations of human LDL as indicated or with 25-OH-CH (3 μg/mL). Cells were evaluated as described under methods for their ability to bind (surface activity) and internalize ^{125}I-LDL. Activity is reported as percentage of LPDS control for each experiment (mean and standard error of six measurements taken over three experiments). See Tables 1 (also for company source) and 2 for abbreviations.

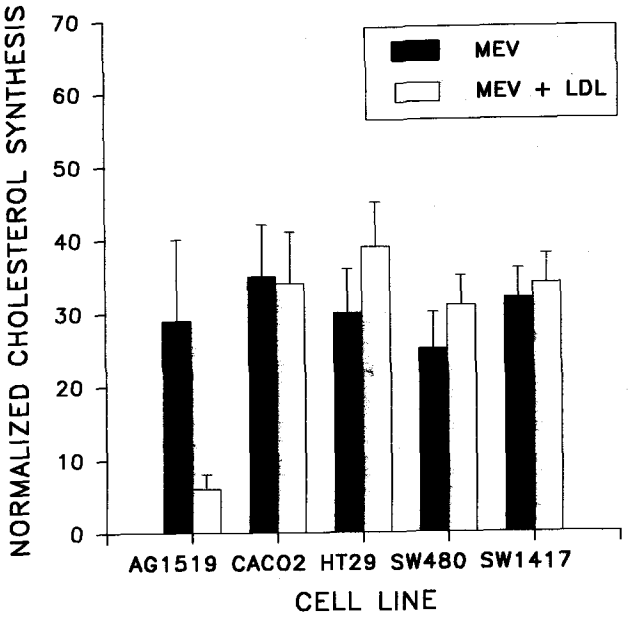


FIG. 3. Cholesterol synthesis in cell lines treated with mevinolin (MEV) or mevinolin and LDL. Cells were cultured as described for Figure 1 and treated with 1 μM mevinolin with (solid bars) or without (open bars) concurrent addition of 50 μg/mL of human LDL. Results are expressed as percentage of LPDS control as described for Figures 1 and 2. See Figure 1 (for company sources also) and 2 for abbreviations.

development of models designed to help elucidate intestinal cholesterol metabolism necessitates embracing the full spectrum of phenotypic characteristics evidenced by such lines. Cholesterol dynamics for a given tumor cell reflect its current state of (i) differentiation which includes not only "poorly vs. well" differentiated characteristics but also the differences between large vs. small bowel phenotypes, and (ii) cellular density in monolayer culture which may or may not affect such differentiation. The colonic adenoCA cell lines HT-29 and $CACO_2$ have shown altered regulation of cholesterol metabolism with the state of differentiation (colonic vs. small intestinal) and confluency (28,41,42). When the adenoCA subline HT-29G is undifferentiated, acyl cholesterol acyl transferase (ACAT) activity was found to be absent (43). Consequently, LDL cannot be directed to this enzyme to be stored as cholesterol esters, or used for lipoprotein synthesis as has been hypothesized in small intestinal cells (39); this could have great impact upon the lipoprotein's ability to affect HMG–CoAR. Mevinolin is known to suppress esterification of exogenous, but not endogenous, cholesterol in enterocytes (see Ref. 58). This suggests an effect of mevinolin on the ACAT substrate pool. The inability of LDL to inhibit synthesis may, therefore, be linked to the ability of a cell to turn ACAT on for storage or lipoprotein synthesis. The latter could reflect a differentiation of the colonic adenoCA cell to a small intestinal phenotype.

The correlation of a decrease in serum cholesterol levels with an increased incidence of colorectal cancer has been the focus of many epidemiologic studies (44–47). Normal human (48) and murine (49) colonic epithelium have been shown to be LDLR positive by immunohistochemistry. Expression of the LDLR was found to vary in different portions of the small and large intestine (37,48,49), which may be imposed by the current state of differentiation (40), and growth (41,50,51) of these cells. In fact, immunohistochemical studies from this laboratory have shown that LDLR expression in human normal and neoplastic colon increases with the degree of differentiation of the tissue (48). Other authors found a high proportion of tumors and surrounding normal mucosa to be negative (52) for LDLR (68% of the cases studied); however, no correlation between LDLR expression and serum cholesterol levels, histological grading, or Duke's stage was found.

Consequently, we investigated the state of cellular density and its effect on LDL regulation of endogenous cholesterol synthesis *in vitro* and found a positive correlation. After three days of culture, LDL was capable of inhibiting cholesterol synthesis in cells growing at low density. In contrast, subconfluent cells cultured for five days did not show this regulation (Fig. 2). We cannot dismiss the fact that the incorporation of ^{14}C-acetate into cholesterol is not a direct measurement of cholesterol synthesis, since differences in intracellular acetate uptake, acetate pools, or activation of acetate to acetyl CoA can occur. Thus, rates of sterol synthesis based on this assay could be regarded as estimates only. Therefore, we conducted a parallel experiment in SW480 cells cultured at the two density states of growth, utilizing ^{14}C-acetate vs. ^{14}C-octanoate as a source of acetyl CoA. Cells preferentially utilize this medium-chain fatty acid as a source of acetyl CoA, *via* its rapid intramitochondrial oxidation, avoiding some of the problems discussed above (53). Results showed that the cells exhibited similar responses to the different treatments when either label was used (data not shown). Nevertheless, since intestinal cells have been reported to prefer ^{14}C-acetate as a source of acetyl CoA to synthesize cholesterol (53), we used ^{14}C-acetate and not ^{14}C-octanoate for our experiments. While it is known that contact inhibited vascular endothelial cells bind LDL with high affinity, without affecting cholesterol metabolism (54), LDLR decrease in number as fibroblasts reach confluency (55). Our results demonstrate that in contrast to the fibroblast, colonic cells exhibited no downregulation of cholesterol synthesis by LDL at subconfluency (Table 1). This variation of cellular density with lipoprotein metabolism also has been shown by many others in intestinal cells in culture (28,40,41) and in neoplastic cells (56,57).

To determine if the culture density-dependent ability of LDL to inhibit cholesterol synthesis was due to a variation in LDLR activity which was concurrently cell density-dependent, the ability of these cultured cell lines to bind and internalize LDL at normal and low densities was assessed. The LDLR activity of LPDS control cultures was 57.3 (normal density) and 89.8 (low density) ng ^{125}I-LDL internalized/mg cell protein. These were not statistically different by ANOVA under even nonstringent conditions ($\alpha = 0.05$; $n = 6$). It is unlikely, should subsequent testing determine an actual difference between these groups, that a 36% decrease in activity from low to normal density should confer an LDL unresponsive phenotype in terms of cholesterol synthesis inhibition. Evidence exists for phenotypic change in terms of cholesterol metabolism with changing growth and differentiation states in colonic adenoCA cells. The observed inability of LDL pretreatment to reduce cholesterol synthesis in four colonic adenoCA cell lines cultured at normal density is similar to observations reported by Villiard *et al.* (58) for HT29 cells in stationary vs. exponential phase. This group also found LDLR activity to be more responsive vs. control under exponential (approximately 85%) than stationary (10%) conditions with a dramatic difference in the differentiated "G-" phenotype (58). This contrasts with our findings, which are supportive of a partially independent regulation of the two sterol controlled genes as suggested previously by others (28,38,40). Under conditions where synthesis of C27 sterols was unaffected by LDL in normal density SW480 cells, LDL pretreatment (5 μg/mL) caused LDLR expression to be reduced to 50 and 34% of the control LPDS treatments in normal and low density cells, respectively. Pretreatment with increased amounts of LDL or 25-OH-CH confirmed that exogenous sterols can be internalized and can cause inhibition of LDLR activity. Although specific degradation of LDL particles was not assessed, the ability of LDL to downregulate its own receptor's activity at each density state is strong evidence that such degradation is taking place even at normal densities where cells are not responsive to cholesterol synthesis inhibition by LDL.

In the fibroblast, the activity of HMG–CoAR and of overall sterol synthesis is regulated according to the rate of cell growth and proliferation (2). However, regulation of the LDLR and HMG–CoAR may not always directly relate to replication, as cell density (59), and standard culture conditions (51) may affect the results. The rates of *de novo* cholesterol synthesis (see Table 1) in cells growing at lower densities were higher than in the subconfluent cells, possibly due to a greater degree of proliferation in the lower density cells. Our laboratory is currently investigating the correlation of LDLR with growth and proliferation in these tumor cells (34).

Treatment of various colonic tumor cell lines with the competitive inhibitor of HMG–CoAR, mevinolin, reduced cholesterol production from acetate in all cells; however, cells cultured at low density were more inhibited by mevinolin treatment than those grown to subconfluence (Fig. 2). Similarly, enhancement of inhibitory action was noted in cells tested at low density relative to subconfluency with other inhibitors (Fig. 2) as 25-OH-CH (nonreceptor-dependent repressor of transcription and enhancer of protein degradation), or LDL (receptor-dependent repressor of transcription and enhancer of protein degradation). These findings are consistent with a possible flux of acetate (and thus mevalonate) toward nonsterol isoprenoids which may be in higher demand in these cells. Inhibitors would thus impact more strongly on cholesterol production. In fact, LDL and mevinolin combined as a treatment in the lower density cells resulted in enhanced inhibition of cholesterol synthesis (Fig. 2) compared to subconfluent densities.

Despite these responses in terms of cholesterol synthetic ability, under these treatments growth is not restored by LDL in intestinal cells inhibited by mevinolin (25,26,28). It is suggested that concurrent treatment with LDL and mevinolin can result in inhibition of HMG–CoAR in such a way that cellular levels of nonsterol mevalonate-derived products may be affected, thereby causing alterations in growth in tumor cells as compared to fibroblasts. This is of considerable interest as the expression of HMG–CoAR is high in tumors of colonic origin (19), and this may affect the expression of active membrane-bound ras oncogene, also found to be high in colon tumors (60–62). Recent work accomplished using a human lung adenoCA cell line has produced similar findings (63); LDL did not reverse a growth inhibition imposed by mevinolin when cells were grown in LPDS. These authors found a high proportion (40%) of HMG–CoAR activity devoted to the generation of nonsterol products in the lung cancer cells, as compared to the 15–20% seen in the fibroblast. Inhibitors of mevalonate synthesis, such as mevinolin, also have proven to inhibit tumor growth in various animal models (64,65). Thus, this inability to utilize exogenous sterol sources for growth in human cells of intestinal origin under conditions of mevalonate inhibition may be of therapeutic significance.

ACKNOWLEDGMENTS

Studies discussed have been supported in part by a research grant CN-95 from the American Cancer Society, and by a training grant T32 CA 09423 and research grant R01 CA57684 from the National Cancer Institute or the National Institutes of Health. The authors acknowledge the helpful suggestions of Dr. Herbert Kupchik. Portions of this work have been presented at the annual meetings of the American Association for Cancer Research (Abstract #79) at Orlando, Florida in March 1993, and (Abstract #2111) at Toronto, Canada, in March 1995.

REFERENCES

1 Chen, H.W. (1984) Role of Cholesterol Metabolism in Cell Growth, *Fed. Proc. 43(1)*, 126–130.
2. Brown, M.S., and Goldstein, J.L. (1986) A Receptor-Mediated Pathway for Cholesterol Homeostasis, *Science 232(4756)*, 34–47.
3. Siperstein, M.D., Gyde, A.M., and Morris, H.P. (1971) Loss of Feedback Control of Hydroxymethylglutaryl Coenzyme A Reductase in Hepatomas, *Proc. Natl. Acad. Sci. USA 68(2)*, 315–317.
4. Betteridge, D.J., Krone, W., Ford, J.M., and Morris, H.P. (1979) Regulation of Sterol Synthesis in Leukaemic Blast Cells: A Defect Resembling Familial Hypercholesterolaemia, *Eur. J. Clin. Invest. 9(6)*, 439–441.
5. Clayman, R.V., Bilhartz, L.E., Buja, L.M., Spady, D.K., and Dietschy, J.M. (1986) Renal Cell Carcinoma in the Wistar-Lewis rat: A Model for Studying the Mechanisms of Cholesterol Acquisition by a Tumor *in vivo*, *Cancer Res. 46(6)*, 2958–2963.
6. Vitols, S., Peterson, C., Larsson, O., Holm, P., and Aberg, B. (1992) Elevated Uptake of Low Density Lipoproteins by Human Lung Cancer Tissue *in vivo*, *Cancer Res. 52(22)*, 6244–6247.
7. Norata, G., Canti, G., Ricci, L., Nicolin, A., Trezzi, E., and Catapano, A.L. (1984) *In vivo* Assimilation of Low Density Lipoproteins by a Fibrosarcoma Tumour Line in Mice, *Cancer Lett. 25(2)*, 203–208.
8. Hynds, S.A. Welsh, J., Stewart, J.M., Jack, A., Soukop, M., McArdle, C.S., Calman, K.C., Packard, C.J., and Shepherd, J. (1984) Low-Density Lipoprotein Metabolism in Mice with Soft Tissue Tumours, *Biochim. Biophys. Acta 795(3)*, 589–595.
9. Lombardi, P., Norata, G., Maggi, F.M., Canti, G., Franco, P., Nicolin, A., and Catapano, A.L. (1989) Assimilation of LDL by Experimental Tumours in Mice, *Biochim. Biophys. Acta 1003(3)*, 301–306.
10. Ho, Y.K., Smith, R.G., Brown, M.S., and Goldstein, J.L. (1978) Low-Density Lipoprotein (LDL) Receptor Activity in Human Acute Myelogenous Leukemia Cells, *Blood 52(6)*, 1099–1114.
11. Gal, D., MacDonald, P.C., Porter, J.C., and Simpson, E.R. (1981) Cholesterol Metabolism in Cancer Cells in Monolayer Culture. III. Low-Density Lipoprotein Metabolism, *Int. J. Cancer 28(3)*, 315–319.
12. Vitols, S., Gahrton, G., Ost, A., and Peterson, C.O. (1984) Elevated Low Density Lipoprotein Receptor Activity in Leukemic Cells with Monocytic Differentiation, *Blood 63(5)*, 1186–1193.
13. Vitols, S., Gahrton, G., Bjorkholm, M., and Peterson, C. (1985) Hypocholesterolaemia in Malignancy Due to Elevated Low-Density-Lipoprotein-Receptor Activity in Tumour Cells: Evidence from Studies in Patients with Leukaemia, *Lancet 2(8465)*:1150–1154.
14. Rudling, M.J., Angelin, B., Peterson, C.O., and Collins, V.P. (1990) Low Density Lipoprotein Receptor Activity in Human Intracranial Tumors and Its Relation to the Cholesterol Requirement, *Cancer Res. 50(3)*:483–487.
15. Rodwell, V.W., Nordstrom, J.L., and Mitschelen, J.J. (1976) Regulation of HMG-CoA Reductase, *Adv. Lipid Res. 14*:1–74.
16. Goldstein, J.L., and Brown, M.S. (1990) Regulation of the Mevalonate Pathway, *Nature 343*, 425–430.
17. Schmidt, R.A., Schneider, C.J., and Glomset, J.A. (1984) Evidence for Post-Translational Incorporation of a Product of

Mevalonic Acid into Swiss 3T3 Cell Proteins, *J. Biol. Chem. 259(16)*, 10175–10180.
18. Maltese, W.A. (1990) Post-Translational Modification of Proteins by Isoprenoids in Mammalian Cells, *FASEB J. 4(15)*, 3319–3328.
19. Maltese, W.A. (1983) 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in Human Brain Tumors, *Neurology 33(10)*, 1294–1299.
20. Engstrom, W., and Schofield, P.M. (1987) Expression of the 3-Hydroxy-3-Methylglutaryl Coenzyme A-Reductase and LDL-Receptor and LDL-Receptor Genes in Human Embryonic Tumours and in Normal Foetal Tissues, *Anticancer Research 7(3 Pt B)*, 337–342.
21. Bennis, F., Favre, G., Le Gaillard, F., and Soula, G. (1993) Importance of Mevalonate-Derived Products in the Control of HMG-CoA Reductase Activity and Growth of Human Lung Adenocarcinoma Cell Line A549, *Int. J. Cancer 55(4)*, 640–645.
22. Goldstein, J.L., and Brown, M.S. (1977) The Low-Density Lipoprotein Pathway and Its Relation to Atherosclerosis, *Ann. Rev. Biochem. 46*, 897–930.
23. Endo, A. (1992) The Discovery and Development of HMG-CoA Reductase Inhibitors, *J. Lipid Res. 33(11)*, 1569–1582.
24. Goldstein, J.L., Helgeson, J.A.S., and Brown, M.S. (1979) Inhibition of Cholesterol Synthesis with Compactin Renders Growth of Cultured Cells Dependent on the Low Density Lipoprotein Receptor, *J. Biol. Chem. 254(12)*, 5403–5409.
25. Fabricant, M., and Broitman, S.A. (1990) Evidence for Deficiency of Low Density Lipoprotein Receptor on Human Colonic Carcinoma Cell Lines, *Cancer Res. 50(3)*, 632–636.
26. Reimann, F.M., Herold, G., Schneider, A., and Stange, E.F. (1991) Compartmentalization of Cholesterol Metabolism and Cellular Growth in Cultured Intestinal Crypt Cells, *Biochim. Biophys. Acta 1085(3)*, 315–321.
27. Fellermann, K., Reimann, F.M., Herold, G., and Stange, E.F. (1992) Mevinolin, A Competitive Inhibitor of Hydroxymethylglutaryl Coenzyme A Reductase, Suppresses Enterocyte Esterification of Exogenous But Not Endogenous Cholesterol, *Biochim. Biophys. Acta 1165(1)*, 78–83.
28. Reimann, F.M., Herold, G., Grosshans, I., Rogler, G., Fellermann, K., and Stange, E.F. (1992) Regulation of Cholesterol Metabolism and Low-Density Lipoprotein Binding in Human Intestinal Caco-2 Cells, *Digestion 51(1)*, 10–17.
29. Viallard, V., Castan, I., Trocheris, V., and Lacombe, C. (1992) Fate of Exogenous and Newly Synthesized Cholesterol in Intestinal Cell Lines, *Int. J. Biochem. 24(8)*, 1315–1321.
30. Zweibaum, A., Pinto, M., Chevalier, G., Bussaulx, E., Triadoo, N., Lacroix, B., Haffen, K., Brun, J.L., and Rousset, M. (1985) Enterocytic Differentiation of a Subpopulation of the Human Colon Tumor Cell Line HT-29 Selected for Growth in Sugar-Free Medium and Its Inhibition by Glucose, *J. Cell Physiol. 122(1)*, 21–29.
31. Chantret, I., Barbat, A., Dussaulx, E., Brattain, M.G., and Zweibaum, A. (1988) Epithelial Polarity, Villin Expression, and Enterocytic Differentiation of Cultured Human Colon Carcinoma Cells: A Survey of Twenty Cell Lines, *Cancer Res. 48(7)*, 1936–1942.
32. Brown, M.S., Faust, J.R., and Goldstein, J.L. (1978) Induction of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Human Fibroblasts Incubated with Compactin (ML-236B), A Competitive Inhibitor of the Reductase, *J. Biol. Chem. 253(4)*, 1121–1128.
33. Goldstein, J.L., Basu, S.K., and Brown, M.S. (1983) Receptor-Mediated Endocytosis of Low-Density Lipoprotein in Cultured Cells, *Methods in Enzymol. 98*, 241–260.
34. Wilkinson, J., and Broitman, S. (1993) Effect of Low Density Lipoprotein (LDL) upon Proliferation in Three Human Adenocarcinoma Cell Lines, in *Proceedings of the American Association for Cancer Research*, Vol. 34, p. 55.
35. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1975) Cholesterol Ester Formation in Cultured Human Fiborblasts. Stimulation by Oxygenated Sterols, *J. Biol. Chem. 250(10)*:4025–4027.
36. Goldstein, J.L., and Brown, M.S. (1974) Binding and Degradation of Low Density Lipoproteins by Cultured Human Fibroblasts. Comparison of Cells from A Normal Subject and from a Patient with Homozygous Familial Hypercholesterolemia, *J. Biol. Chem. 249(16)*, 5153–5162.
37. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1973) Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Human Fibroblasts by Lipoproteins, *Proc. Natl. Acad. Sci. (USA) 70(7)*, 2162–2166.
38. Stange, E., and Dietschy, J.M. (1983) Cholesterol Synthesis and Low Density Lipoprotein Uptake Are Regulated Independently in Rat Small Intestinal Epithelium, *Proc. Natl. Acad. Sci. (USA) 80(18)*, 5739–5743.
39. Safanova, I.G., Sviridov., D.D., Roytman, A., Rytikov, F.M., Dolgov, V.V., Nano, J.L., Rampal, P., and Repin, V.S. (1993) Cholesterol Uptake in the Human Intestine. Hypo- and Hyperresponsivenuess, *Biochim. Biophys. Acta 1166(2–3)*, 313–316.
40. Suckling, K.E., and Stange, E.F. (1985) Role of Acyl-CoA: Cholesterol Acyltransferase in Cellular Cholesterol Metabolism, *J. Lipid Res. 26*, 647–671.
41. Viallard, V., Lacombe, C., Trocheris, V., Tabacik, C., and Aliau, S. (1990) Metabolism of Low-Density Lipoprotein in Differentiated and Undifferentiated HT29 Colon Cancer Cells, *Int. J. Cancer 46(2)*, 320–325.
42. Nano, J.L., Barbaras, R., Negrel, R., and Rampal, P. (1986) Regulation of Cholesterol Synthesis and Binding of Lipoproteins in Cultured Rat Intestinal Epithelial Cells, *Biochim. et Biophys. Acta 876(1)*, 72–789.
43. Viallard, V., Lacombe, C., Nebot, C., and Castan, I. (1991) Acyl-CoA: Cholesterol Acyltransferase in HT 29 Cell Subpopulations. Defect of Activity in the Undifferentiated Cells, *Biochim. Biophys. Acta 1085(2)*, 265–271.
44. Broitman, S.A. (1986) Dietary Cholesterol, Serum Cholesterol, and Colon Cancer: A Review, *Advances in Experimental Medicine and Biology* (Poirier, L.A., Newberne, P.M., and Pariza, M.W., eds.) pp. 137–182, Plenum Press, New York.
45. Winawer, S.J., Flehinger, B.J., Buchalter, J., Herbert, E., and Shike, M. (1990) Declining Serum Cholesterol Levels Prior to Diagnosis of Colon Cancer. A Time-Trend, Case-Control Study, *JAMA 263(15)*, 2083–2085.
46. Cowan, L.D., O'Connell, D.L., Criqui, M.H., Barrett-Connor, E., Bush, T.L., and Wallace, R.B. (1990) Cancer Mortality and Lipid and Lipoprotein Levels. Lipid Research Clinics Program Mortality Follow-up Study, *Am. J. Epiodemiol. 131(3)*:468–482.
47. Kreger, B.E., Anderson, K.M., Schatzkin, A., and Splansky, G.L. (1992) Serum Cholesterol Level, Body Mass Index, and the Risk of Colon Cancer. The Framingham Study, *Cancer 70(5)*, 1038–1043.
48. Sotnikov, A.V., Kupchik, H.Z., Gottlieb, L.S., Steele, G., and Broitman, S.A. (1992) *Gastroenterology 102*, A400.
49. Fong, L.G., Bonney, E., Kosek, J., and Cooper, A.D. (1989) Immunohistochemical Localization of Low Density Lipoprotein Receptors in Adrenal Gland, Liver, and Intestine, *J. Clin. Invest. 84(3)*, 847–856.
50. Mazzone, T., Basheeruddin, K., Ping, L., and Schick, C. (1990) Relation of Growth- and Sterol-Related Regulatory Pathways for Low Density Lipoprotein Receptor Gene Expression, *J. Biol. Chem. 265(9)*, 5145–5149.
51. Tavangar, K., and Kraemer, F.B. (1988) The Regulation of Hydroxymethylglutaryl-CoA Reductase in Cultured Cells, *Biochim. Biophys. Acta 970(3)*, 251–261.

52. Caruso, M.G., Notarnicola, M., Cavallini, A., Guerra, V., Misciagna, G., and Di Leo, A. (1993) Demonstration of Low Density Lipoprotein Receptor in Human Colonic Carcinoma and Surrounding Mucosa by Immunoenzymatic Assay, *Ital. J. Gastroenterol. 25(7)*, 361–367.
53. Stange, E.F., and Dietschy, J.M. (1983) Absolute Rates of Cholesterol Synthesis in Rat Intestine *in vitro* and *in vivo*: A Comparison of Different Substrates in Slices and Isolated Cells, *J. Lipid Res. 24(1)*, 72–82.
54. Vlodavsky, I., Fielding, P.E., Fielding, C.J., and Gospodarowicz, D. (1978) Role of Contact Inhibition in the Regulation of Receptor-Mediated Uptake of Low Density Lipoprotein in Cultured Vascular Endothelial Cells, *Proc. Natl. Acad. Sci. (USA) 75(1)*, 356–360.
55. Kruth, H.S., Avigan, J., Gamble, W., and Vaughan, M. (1979) Effect of Cell Density on Binding and Uptake of Low Density Lipoprotein by Human Fibroblasts, *J. Cell Biol. 83(3)*, 588–594.
56. Gal, D., MacDonald, P.C., Porter, J.C., Smith, J.W., and Simpson, E.R. (1981) Effect of Cell Density and Confluency on Cholesterol Metabolism in Cancer Cells in Monolayer Culture, *Cancer Res. 41(2)*, 473–477.
57. Rotheneder, M., and Kostner, G.M. (1989) Effects of Low- and High-Density Lipoproteins on the Proliferation of Human Breast Cancer Cells *in vitro*: Differences Between Hormone-Dependent and Hormone-Independent Cell Lines, *Int. J. Cancer 43(5)*, 875–879.
58. Villiard, V., Lacombe, C., Trocheris, V., Tabacik, C., and Aliau, S. (1990) Metabolism of Low-Density Lipoprotein in Differentiated and Undifferentiated Ht 29 Colon Cancer Cells, *Int. J. Cancer 46(2)*, 320–325.
59. Kenagy, R., Bierman, E.L., and Schwartz, A. (1983) Regulation of Low-Density Lipoprotein Metabolism by Cell Density and Proliferative State, *J. Cell Physiol. 116(3)*, 404–408.
60. Michelassi, F., Leuthner, S., Lubienski, M., Bostwick, D., Rodgers, J., and Handcock, M. (1987) Ras Oncogene p21 Levels Parallel Malignant Potential of Different Human Colonic Benign Conditions, *Arch. Surg. 122(12)*, 1414–1416.
61. Hand, PH, Thor, A., Wunderlich, D., Muraro, R., Caruso, A., and Schlom, J. (1984) Monoclonal Antibodies of Predefined Specificity Detect Activated *ras* Gene Expression in Human Mammary Carcinomas, *Proc. Natl. Acad. Sci. 81*, 5227–5231.
62. Hand, P.H., Thor, A., Wunderlich, D., Muraro, R., Caruso, A., and Schlom, J. (1984) *Proc. Natl. Acad. Sci. (USA) 81*, 5227–5231.
63. Bennis, F., Favre, G., Le Gaillard, F., and Soula, G. (1993) Importance of Mevalonate-Derived Products in the Control of HMG-CoA Reductase Activity and Growth of Human Lung Adenocarcinoma Cell Line A549, *Int. J. Cancer 55(4)*, 640–645.
64. Maltese, W.A., Defendini, R., Green, R.A., Sheridan, K.M., and Donley, D.K. (1985) Suppression of Murine Neuroblastoma Growth *in vivo* by Mevinolin, A Competitive Inhibitor of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase, *J. Clin. Invest. 76(5)*, 1748–1754.
65. Sumi, S.R., Beauchamp, R.D., Townsend, C.M., Uchida, T., Murakami, M., Rajaraman, S., Ishizuka, J., and Thompson, J.C. 91992) Inhibition of Pancreatic Adenocarcinoma Cell Growth by Lovastatin, *Gastroenterology 103(3)*, 982–989.

[Received December 30, 1994, and in final revised form September 21, 1995; Revision accepted October 20, 1995]

Functional and Ultrastructural Effects of Essential Fatty Acid Deficiency in Kidney Epithelial Cells

Lynn L. Stoll* and Arthur A. Spector

Departments of Anesthesia and Biochemistry, University of Iowa, Iowa City, Iowa 52242

ABSTRACT: Madin-Darby canine kidney (MDCK) epithelial cells were grown in culture medium supplemented with 1% fetal bovine serum (FBS) to provide a cell culture model of essential fatty acid deficiency (EFAD). 5,8,11-Eicosatrienoic acid (20:3n-9) accumulated in cellular phospholipids, and arachidonic acid (20:4) decreased. A large increase in cellular cholesterol/phospholipid ratio was observed. Hemicyst formation was greatly reduced from normal levels in the EFAD–MDCK cells. Scanning and transmission electron microscopy revealed that EFAD–MDCK were much flatter than their normal counterparts. They had much less dense surface microvilli, mitochondria and other organelles were very sparse, except in the perinuclear area, and much of the peripheral cytoplasm was amorphous. The EFAD was rapidly reversed by the addition of as little as 10 μM linoleic or arachidonic acid to the medium. Cells supplemented with 10% FBS, the usual culture condition, displayed borderline EFAD, with intermediate levels of 20:3n-9 and 20:4 and hemicyst formation. These studies suggest that EFAD reduces water and electrolyte transport in renal tubular epithelium.

Lipids 30, 1093–1103 (1995).

Essential fatty acid deficiency (EFAD) is known to produce metabolic and functional disturbances in a wide variety of cells and tissues. These disturbances include a decrease in macrophage leukotriene production (1) and acetylated low density lipoprotein receptor activity (2), impaired intestinal fat absorption (3), decreased Ca^{2+}-ATPase activity in submandibular gland microsomes (4), and decreased growth, cessation of beating, and impaired oxidative phosphorylation in cardiac myocytes (5).

Impaired renal function has long been recognized as one of the hallmarks of EFAD (6). The condition has been extensively characterized in animal models, with the accumulation of 5,8,11-eicosatrienoic acid (20:3n-9) and an increase in the ratio of fatty acid trienes/tetraenes considered diagnostic (6,7). EFAD in the rat produces a salt-sensitive hypertension with a combined defect in both sodium and water excretion which is related to prostaglandin depletion (8). Administration of 0.5 g/d of linoleic acid to these essential fatty acid-deficient rats normalized sodium and water excretion and returned urinary prostaglandin E output to normal within 3–4 d. Strandvik *et al.* (9) have shown that EFAD can be found in patients with cystic fibrosis and may contribute to the pathophysiology of the disease, as intravenous administration of essential fatty acids normalized the increased glomerular filtration rate found in these patients (10). EFAD also has been found in chronic hemodialysis patients, where it is associated with erythrocyte fragility, skin problems, and other clinical abnormalities (11).

Laposata *et al.* (12) have reported the development and characterization of a tissue culture model system for EFAD derived from the $HSDM_1C_1$ mouse fibrosarcoma cell line. These authors showed that the phenotypic modulation observed in this line can be reversed by the addition of arachidonic acid, but not by any other fatty acid that was tested (13). Although this line is useful for studies of cell growth and lipid metabolism, the mouse fibrosarcoma does not exhibit any characteristic physiological function. Madin-Darby canine kidney (MDCK) cells are widely used as an *in vitro* renal distal tubule model system for studying various aspects of renal function (14,15). We now report the development of a line of MDCK cells grown in medium supplemented with only 1% fetal bovine serum (FBS), which display many of the characteristics of EFAD seen in animal models. These include accumulation of eicosatrienoic acid (20:3n-9) and probably of its elongation product, docosatrienoic acid (22:3n-9), altered gross and ultrastructural morphology, growth inhibition, reduced transport function as indicated by decreased hemicyst formation, and changes in the cell phospholipid-to-cholesterol ratio, suggesting membrane abnormalities. Our results with this EFAD–MDCK cell culture model indicate that EFAD reduces water and electrolyte transport in the renal tubule, as indicated by decreased hemicyst formation.

MATERIALS AND METHODS

Cell culture. MDCK cells (ATCC CCL 34) were obtained from the American Type Culture Collection (Rockville, MD). Stock cultures were maintained in minimum essential medium (MEM) with Earle's Salts (GIBCO, Grand Island,

*To whom correspondence should be addressed at 2-317 BSB, Department of Anesthesia, The University of Iowa, Iowa City, IA 52242.

Abbreviations: EFAD, essential fatty acid deficiency; FBS, fetal bovine serum; MDCK, Madin-Darby canine kidney; MEM, minimum essential medium.

Copyright © 1995 by AOCS Press

NY) supplemented with MEM Nonessential Amino Acids (GIBCO), MEM Vitamin Solution (GIBCO), HEPES (Sigma, St. Louis, MO), 2 mM L-glutamine (Sigma), 50 μg/mL gentamicin (Schering Corp., Kenilworth, NJ), and either 1, 10, or 20% HyClone Defined Fetal Bovine Serum (HyClone, Logan, UT). For electron microscopy studies, cells were grown in either 20% FBS or 1% FBS plus 19% delipidized FBS prepared according to the method of Rothblat *et al.* (16). The EFAD–MDCK subline had been adapted to growth in the medium containing 1% FBS plus 19% delipidized serum for at least three passages before being used for experiments. Cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2. Stocks were subcultured weekly by trypsinization and reseeding at a 1:5 dilution for cells grown in 1% FBS, 1:10 for cells grown in 10% FBS, and 1:20 for cells grown in 20% FBS. Fatty acid-supplemented incubation media were prepared by adding the sodium salt of each fatty acid to medium containing 1% FBS (17).

Phospholipid fatty acyl composition. Confluent cell monolayers were washed three times with cold, phosphate-buffered saline solution (pH 7.4), and then harvested by scraping with a rubber policeman. The cells were pelleted by centrifugation; the cell pellet was resuspended in cold buffer and the cell lipids extracted with chloroform/methanol (2:1, vol/vol). Phospholipids were isolated by thin-layer chromatography on Silica Gel G plates (Alltech Associates, Deerfield, IL) using a solvent system of mixed hexanes/diethyl ether/acetic acid/methanol (170:40:4:4, by vol). The phospholipids were eluted from the gel with chloroform/methanol (1:1, vol/vol) and then saponified and methylated (17). Fatty acid methyl esters were separated by gas–liquid chromatography using a Hewlett-Packard (Avondale, PA) Model 5700 gas–liquid chromatograph with a 1.8 m glass column (2-mm i.d.) packed with 10% SP-2330 on 100/120 mesh Chromosorb W-AW (Supelco, Bellefonte, PA). Peak assignments were made by comparing retention times with those of fatty acid standards (Nu-Chek-Prep, Elysian, MN). Peak areas were determined with a Hewlett-Packard Model 3380-S integrator-recorder and are reported as weight percentages.

Mass spectrometry. Fatty acids were collected using a gas–liquid chromatograph equipped with a 9:1 stream splitter, with 90% of the effluent being diverted through Teflon tubing into CS_2. Approximately 1 μg of the collected fatty acid methyl ester was introduced into the solid inlet probe of a Nermag R10-10C mass spectrometer coupled to a Nermag Model 40 Data System (Delsi-Nermag, Paris, France). Conditions employed were a 25 m column of 5% phenyl methyl silicone, inner diameter 0.2 mm, film thickness 0.33 μm, temperature 220°C, and pressure 1 bar. Ionization was by electron impact at 70 eV.

Phospholipid classes. Individual phospholipid classes were separated from the chloroform/methanol extracts of the cell lipids by thin-layer chromatography, using boric acid-impregnated Silica Gel G plates and a solvent system of $CHCl_3/H_3OH/NH_4OH/H_2O$ (120:75:2:6, by vol) (18). Appropriate standards were cochromatographed on each plate. Phospholipids of each class were eluted from the gel with chloroform/methanol (1:1, vol/vol).

Processing of cells for scanning electron microscopy. MDCK and EFAD–MDCK cells were grown in Falcon P-35 plastic tissue culture dishes. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After three buffer rinses over a 15-min period, the fixed cells were treated with freshly prepared 1% OsO_4 for 30 min. This was followed by a thorough rinsing with 5–10 changes of 0.1 M sodium cacodylate buffer. Cells were then treated with 1% tannic acid in 0.1 M cacodylate buffer for 1 h, followed by a rinse with glass-distilled water. The initial dehydration step was in 50% ethanol for 15 min; the cells were then stained overnight with 2.5% uranyl acetate in 75% ethanol. The following morning they were rinsed several times with 75% ethanol, and then dehydration was continued with 95% ethanol for 15 min and finally 100% ethanol. At each step, care was taken not to remove all of the fluid from the cells, so as to prevent drying and cracking of the monolayer. Sections of the plastic tissue culture dish with dehydrated cells were cut out and critical point dried, mounted on stubs, coated with gold in an Emscope SC-500 sputter coater (Ashford, Kent, England), and examined in a Jeol (Tokyo, Japan) 35C scanning electron microscope.

Processing of cells for transmission electron microscopy. Confluent cultures of both MDCK and EFAD–MDCK cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After three buffer rinses over a 15-min period, the fixed cells were treated with freshly prepared 1% OsO_4 for 30 min; this was followed by two buffer rinses and one distilled water rinse. Cells were then stained for 5 min with 2.5% uranyl acetate. Cells were dehydrated with successive 10-min treatments in 25, 50, and 75% ethanol, followed by 20 min in 95% ethanol and 3 × 20 min in 100% ethanol. After dehydration, cells were infiltrated with Epon (Ted Pella, Inc., Redding, CA) (1:1 Epon/ethanol for 30–60 min, followed by 1 h in 100% Epon). Ultrathin sections were stained with 5% uranyl acetate for 10 min, rinsed well, stained with 0.5% lead citrate for 6–8 min, rinsed again, allowed to air-dry, and examined in a Hitachi (Toyko, Japan) H-600 transmission electron microscope.

Other determinations. Lipid phosphorous was determined by the method of Chalvardjian and Rudnicki (19). Proteins were measured by the Lees and Paxman modification of the Lowry procedure (20). Cholesterol content was determined according to the Manual of Laboratory Operations, Lipid Research Clinics Program (21).

RESULTS

Identification of 5,8,11-eicosatrienoic acid. 5,8,11-Eicosatrienoic acid (20:3n-9) accumulated in MDCK cell lipids under certain conditions, as identified by its elution on gas–liquid chromatograms (between 20:2n-6 and 20:3n-6) (Fig. 1) and by mass spectrometry (Fig. 2, top panel). The mass spectrum of the methyl ester contained a molecular ion with *m/z* 320, consistent with a structure of 20:3. Other ions

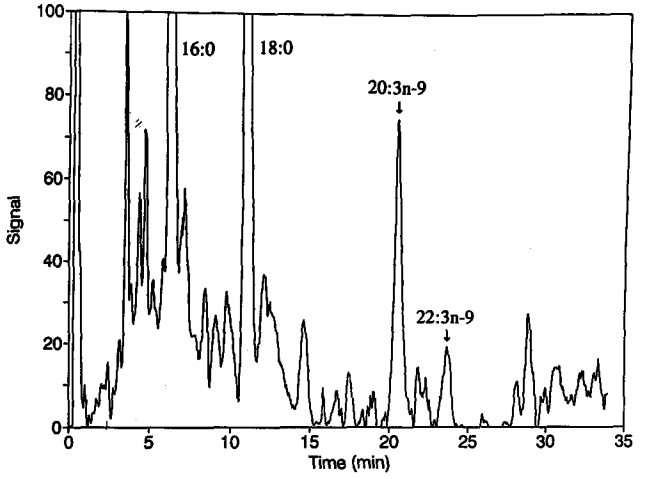


FIG. 1. Gas–liquid chromatogram, showing retention times and relative composition of 5,8,11-eicosatrienoic acid (20:3n-9), retention time 20.55 min, and putative 7,10,14-docosatrienoic acid (22:3n-9), retention time 23.72 min, in essential fatty acid deficiency–Madin-Darby canine kidney cells. Retention times for other fatty acids in this system: 20:2n-6, 19.45 min; 20:3n-6, 22.04 min; 20:4, 22.76 min; 24:0, 28.18; 24:1, 28.96.

consistent with this structure are m/z = 221, M − 99 (loss of C_7H_{15}); m/z = 219, M − 101 (loss of $C_5H_9O_2$); m/z = 161, $C_{12}H_{17}$; m/z = 147, $C_{11}H_{15}$; m/z = 135, $C_{10}H_{15}$; m/z = 120, C_9H_{12}; m/z = 106, C_8H_{10}; m/z = 80, C_6H_8; and m/z = 67, C_5H_7. This fragmentation pattern is identical to a mass spectrum obtained in a previous study of 20:3n-9 accumulation in 3T3-L1 and 3T3 cells (22).

A smaller peak which eluted on gas–liquid chromatography between 20:5n-3 and 22:4n-6 (Fig. 1) also was collected and analyzed by mass spectrometry. The mass spectrum from this fatty acid methyl ester revealed a molecular ion of m/z 348 (Fig. 2, bottom panel), consistent with a structure of 22:3, possibly the elongation product of 20:3n-9. Other observed ions that are consistent with the 22:3 structure are m/z = 317, M − 31 (loss of OCH_3); m/z = 249, M − 99 (loss of C_7H_{15}); m/z = 208, M − 140 (loss of $C_{10}H_{20}$); m/z = 192, M − 156 (loss of $C_9H_{16}O_2$); m/z = 163, $C_{12}H_{33}$; m/z = 149, $C_{11}H_{19}$; m/z = 135, $C_{10}H_{15}$; m/z = 121, C_9H_{13}; m/z = 107, C_8H_{11}; m/z = 94, C_7H_{10}; m/z = 87, $C_4H_7O_2$; m/z = 80, C_6H_8; m/z = 71, C_5H_{11}; and m/z = 67, C_5H_7. Further studies to unequivocally assign double bond locations were not performed.

Serum concentration and fatty acid composition. Experiments with MDCK cells grown in media supplemented with FBS in concentrations ranging from 0 to 20% indicate an inverse relationship between 20:3n-9 and arachidonic acid (20:4n-6) content in cell phospholipids (Table 1). In cells which had been grown for several passages in medium supplemented with delipidized serum only, the cell phospholipids contained 9.0 ± 0.39% 20:3n-9, with no detectable 20:4 or other n-6 polyunsaturated fatty acids, 18:2n-6 and 20:3n-6. We were unable to maintain MDCK cells under these conditions for more than six passages. However, addition of only 1% FBS along with 19% delipidized serum to the growth

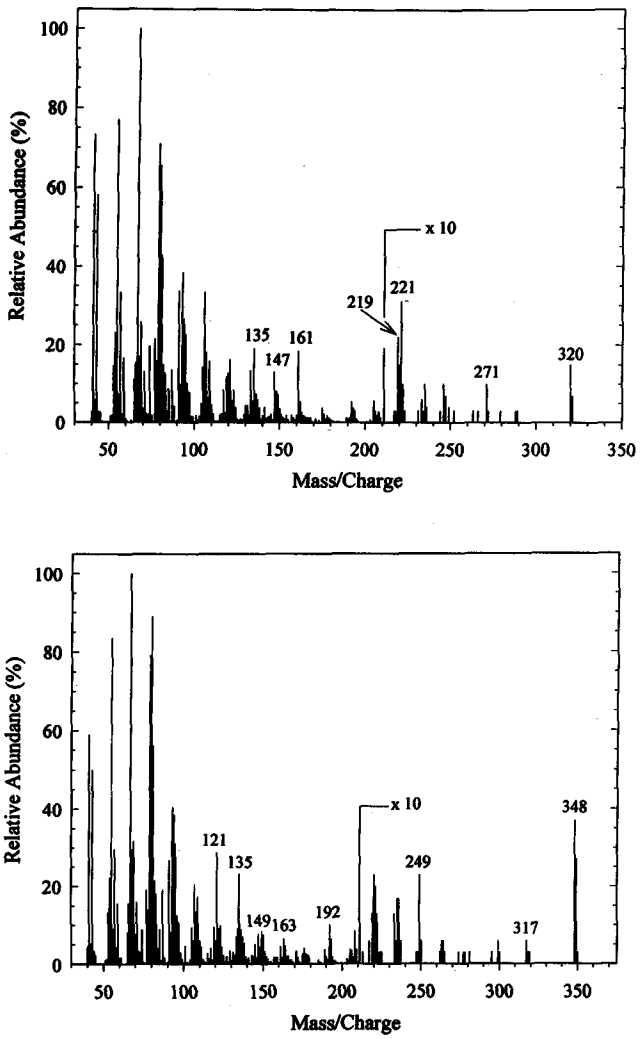


FIG. 2. Electron impact mass spectra of 5,8,11-eicosatrienoic acid (20:3n-9) (top panel) and a fatty acid suggested to be 7,10,13-docosatrienoic acid (bottom panel). Fatty acid methyl esters were isolated by collecting the effluent from the peaks using a gas–liquid chromatograph equipped with a 9:1 stream-splitter. These spectra are consistent with the structures of 20:3 and 22:3, respectively.

medium permitted maintenance up to 20 passages, with the phospholipid 20:3n-9 content ranging from 6–8% in several experiments, and the 20:4 content remaining at approximately 1%. Cells maintained under these conditions are clearly essential fatty acid-deficient by the traditional criterion of fatty acid triene/tetraene ratio = >1 (6), exhibiting a triene/tetraene ratio ranging from 3.1 to 10.9 in several experiments. The putative 22:3n-9 peak was detected only in cells grown in medium containing 5% or less FBS.

MDCK cells grown in a medium containing 10% FBS, the usual method of culture, contained approximately equal percentages of 20:3n-9 and 20:4 in phospholipids. The fatty acid triene/tetraene ratio, which is approximately 1, indicates that MDCK cells grown under ordinary culture conditions actually are in a state of borderline EFAD (6). A further increase in serum concentration above 10% substantially reduced the 20:3n-9 content, with a concomitant increase in

TABLE 1
Fatty Acid Composition of Cellular Phospholipids[a]

Fatty acid	Phospholipid fatty acid composition					
	Serum-free	1%[b]	5%	10%	15%	20%
16:0	12.3 ± 0.32	12.1 ± 0.92	11.3 ± 0.23	14.2 ± 0.49	14.6 ± 1.04	13.2 ± 0.52
16:1	8.2 ± 0.26	6.3 ± 0.74	5.1 ± 0.03	4.7 ± 0.20	4.6 ± 0.36	3.9 ± 0.03
18:0	15.6 ± 0.30	18.6 ± 1.35	15.1 ± 0.06	15.3 ± 0.26	16.6 ± 0.18	16.7 ± 0.18
18:1	38.7 ± 0.95	43.5 ± 0.5	44.1 ± 0.33	41.7 ± 0.70	42.3 ± 1.02	42.2 ± 1.61
18.2	nd[c]	nd	2.4 ± 0.14	1.8 ± 0.39	2.0 ± 0.15	2.0 ± 0.18
20:3n-9	9.0 ± 0.39	6.9 ± 0.70	6.4 ± 0.09	5.5 ± 0.12	3.1 ± 0.09	3.4 ± 0.14
20:3n-6	nd	0.3 ± 0.03	0.9 ± 0.06	1.1 ± 0.07	1.2 ± 0.09	1.4 ± 0.05
20:4	nd	0.8 ± 0.49	3.5 ± 0.13	4.1 ± 0.10	5.1 ± 0.32	6.2 ± 0.14
22:3n-9	1.5 ± 0.03	1.5 ± 0.35	1.1 ± 0	nd	nd	nd

[a]Madin-Darby canine kidney cells were grown for seven days in either serum-free medium or in medium supplemented with the indicated percentages of serum. After the lipids were extracted and the phospholipids isolated by thin-layer chromatography, the fatty acid composition of the phospholipids was determined by gas–liquid chromatography. Each value is the mean ± SEM of three separate cultures.
[b]Percent fetal bovine serum in culture medium.
[c]nd = Not detected.

20:4 content. Medium supplemented with 20% FBS produces a normal phospholipid fatty acid triene/tetraene ratio of approximately 0.5.

Culture morphology. Coincident with these fatty acid compositional changes was the observation of greatly enhanced hemicyst formation in cultures grown with 20% FBS supplementation as compared to cultures grown with lower percentages of FBS (Fig. 3). A hemicyst is a blister-like phenomenon formed when renal tubule epithelial cells grown on tissue culture plastic transport water and salt from the apical to the basolateral surface. This fluid is trapped under the cell layer, rather than being released through the tubule, causing the formation of groups of cells rising in domes above the plane of the cell surface. Loss of hemicyst formation is attributed to loss of transport function by the epithelium (23). EFAD–MDCK cultures grown with 1% FBS supplementation (Fig. 3, panel A) exhibited a virtual absence of hemicysts, with only one hemicyst observed in a total of 25 consecutive low-power fields examined. With 10% FBS supplementation, a total of nine hemicysts were observed in a series of 25 consecutive lower power fields examined. However, with cells grown with 20% supplementation, a mean of 31 ± 1.9 hemicysts were observed in each low-power field. Changes in gross morphology were also evident, with the EFAD–MDCK being flattened and spread compared to the cells grown in 10 or 20% FBS.

Fatty acid supplementation. The EFAD of EFAD–MDCK grown with 1% FBS supplementation was reversed within a single passage by the addition of either linoleic acid (18:2n-6), a precursor of arachidonic acid, or arachidonic acid

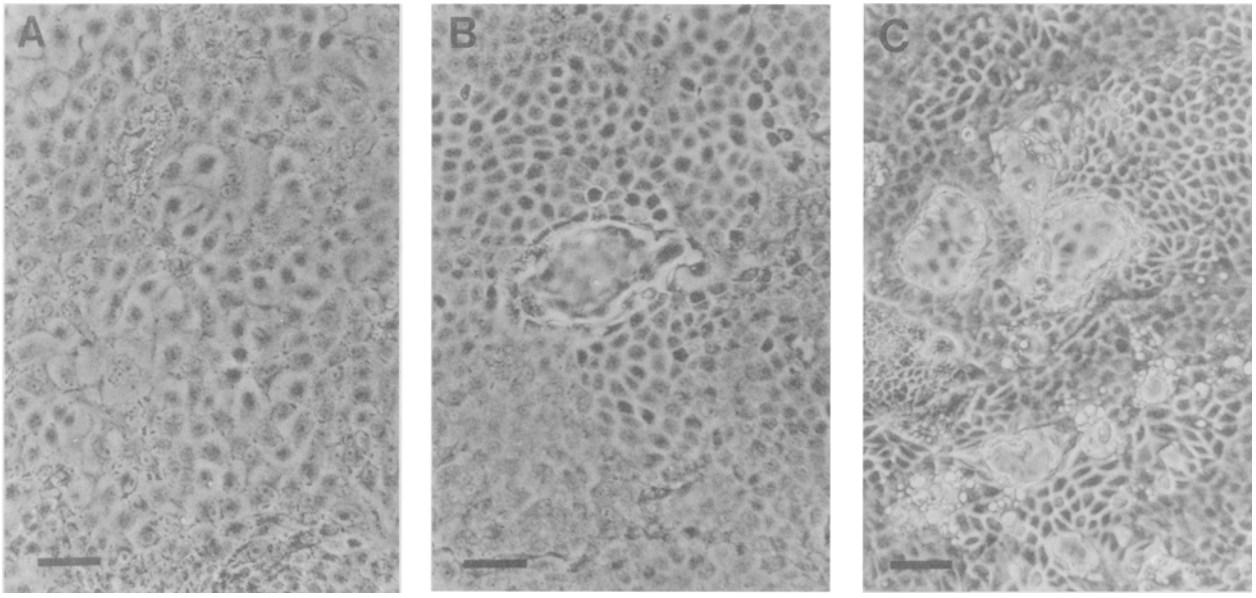


FIG. 3. Phase contrast photomicrographs of Madin-Darby canine kidney cells grown in medium supplemented with 1% (Panel A), 10% (Panel B), and 20% (Panel C) fetal bovine serum; bar = 100 μm.

TABLE 2
Effect of Increasing Concentrations of Supplemental 18:2 or 20:4 on the Phospholipid Fatty Acid Composition of Madin-Darby Canine Kidney Cells Grown in 1% Fetal Bovine Serum[a]

	Phospholipid fatty acid composition (%)						
		18:2[b]			20:4[c]		
Fatty acid	Control	10 μM	25 μM	50 μM	10 μM	25 μM	50 μM
16:0	12.2 ± 0.03[d]	13.3[e]	11.2	10.6	11.4	12.4	13.2
16:1	6.3 ± 0.41	4.7	2.8	2.4	4.1	3.4	2.6
18:0	17.4 ± 0.32	17.0	20.2	20.2	18.8	23.8	22.6
18:1	44.9 ± 0.78	38.2	32.4	27.4	42.8	37.6	25.2
18:2	<0.1	5.1	9.0	13.0	2.2	1.7	1.8
20:3n-9	4.6 ± 0.11	2.9	1.6	1.0	2.8	0.8	0.2
20:3n-6	0.3 ± 0.02	3.2	5.8	6.2	1.1	1.4	2.6
20:4	1.3 ± 0.12	3.8	9.4	11.2	7.8	11.8	22.2
22:4	0.3 ± 0.13	0.9	1.0	1.4	1.5	1.8	2.8

[a]Madin-Darby canine kidney cells were grown for seven days in medium containing 1% FBS (control) or in this medium supplemented with the indicated amounts of either linoleic (18:2) or arachidonic (20:4) acid.
[b]Media supplemented with linoleic acid.
[c]Media supplemented with arachidonic acid.
[d]Mean ± SEM (n = 3).
[e]Average of two determinations from separate cultures.

itself to the growth medium (Table 2). In this experiment, the ratio of 20:3n-9 to 20:4 in the phospholipids of control cells grown with 1% FBS supplementation was approximately three; this was reduced to <1 by the addition of as little as 10 μM of either 18:2 or 20:4. The phospholipid content of 20:3n-9 was reduced to 1% by supplementation with 50 μM linoleic acid, and virtually eliminated by supplementation with 50 μM arachidonic acid. In addition, the amounts of palmitoleic (16:1n-7) and oleic (18:1n-9) acids were substantially reduced by these treatments.

A generally similar, though less complete, reversion of the EFAD pattern occurred when confluent cultures of EFAD–MDCK were placed in medium containing 10 or 20% FBS or 10% horse serum. FBS is rich in arachidonic acid, and horse serum is rich in linoleic acid (24). Table 3 shows the alterations that take place in the phospholipid fatty acid composition at different times up to seven days after changing from 1% FBS supplementation to these media that contain a higher serum content. As early as 6 h after changing the culture medium, the phospholipid 20:4 content increased from 1% to 2.7 ± 0.17% with 10% FBS, and 3.2 ± 0.19% with 20% FBS. After seven days, 20:4 levels increased to 3.8 ± 0.23% with 10% FBS and 4.9 ± 0.21 with 20% FBS. However, 20:3n-9 decreased much more slowly. At seven days, 20:3n-9 levels decreased from an initial value of 6.8 ± 0.26% to 5.5 ± 0.22% in 10% FBS, and 3.6 ± 0.21% in 20% FBS.

Hemicyst formation. A significant number of hemicysts began to appear in cells three days after changing from 1 to 20% FBS supplementation, at which time 20:3n-9 and 20:4 contents were 5.0 ± 0.12 and 3.4 ± 0.22%, respectively. Seven days after adding 20% FBS-supplemented medium, hemicyst formation was equivalent to that seen in cells grown continuously with 20% FBS supplementation, suggesting that the presence of phospholipid 20:3n-9 does not itself inhibit hemicyst formation. Hemicyst formation also was rapidly induced by supplementation of the culture medium of EFAD–MDCK with 18:2 or 20:4, but not with 18:1 (Table 4). Before the fatty acids were added to confluent cultures of EFAD–MDCK, there were 2–3 hemicysts per 25 low-power fields. After 48 h this increased to 157 hemicysts per 25 fields in the 18:2-supplemented cells, and 73 per 25 fields in the 20:4-supplemented cultures. By contrast, supplementation with 10 μM 18:1 for 48 h had little effect.

Lipid composition. Determination of total lipid phosphorous and its distribution among different phospholipid classes revealed marked differences between the EFAD–MDCK and the MDCK cells grown with 10 or 20% FBS supplementation. There was a sharp reduction in the amount of total lipid phosphorous per mg cell protein in the EFAD–MDCK (Table 5). Furthermore, the relative amounts of the individual phospholipid classes differed (data not shown). Of the three major phospholipid classes analyzed, which accounted for 79–89% of the total phospholipids, only phosphatidylinositol increased in the EFAD–MDCK, both in absolute amount and especially in percentage (from 8 to 17%). Phosphatidylcholine decreased substantially both in absolute amount and in percentage (from 49 to 34%). Phosphatidylethanolamine declined in absolute amount, but remained a constant percentage of the total phospholipids (approximately 30%). In contrast to the total lipid phosphorous values, the total cholesterol content of the EFAD–MDCK cells was not significantly different from that of the MDCK cells grown with 10 or 20% FBS supplementation (Table 5). Therefore, the cholesterol/phospholipid molar ratio was significantly higher in the EFAD–MDCK cells compared to the two other groups.

Scanning electron microscopy. The morphological differences between EFAD–MDCK and normal MDCK cells observed by light microscopy (Fig. 3) were confirmed by scanning electron microscopy. Pairs of micrographs (Fig. 4, panels A vs. C, and B vs. D of MDCK and EFAD–MDCK at the same

TABLE 3
Changes in Phospholipid Fatty Acid Composition of Essential Fatty Acid Deficient–Madin-Darby Canine Kidney Cells Grown in Medium Supplemented with 10 or 20% Fetal Bovine Serum (FBS) or 10% Horse Serum

	Phospholipid fatty acid composition (%)				
Fatty acid	0	6 h	1 d	3 d	7 d
Control (1% FBS)					
16:0	11.0 ± 0.77[a]				
16:1	6.0 ± 0.77				
18:0	16.3 ± 0.71				
18:1	49.4 ± 1.53				
18:2	nd[b]				
20:3n-9	6.8 ± 0.26				
20:3n-6	nd				
20:4	1.2 ± 0.08				
22:3n-9	1.3 ± 0.06				
10% FBS					
16:0		12.9 ± 0.77	12.5 ± 1.10	14.3 ± 0.33	15.9 ± 0.47
16:1		5.8 ± 0.63	5.0 ± 0.59	5.9 ± 0.44	6.3 ± 0.55
18:0		16.1 ± 0.25	16.2 ± 0.29	15.7 ± 0.44	13.7 ± 0.19
18:1		47.2 ± 0.59	47.1 ± 0.87	46.1 ± 1.36	43.3 ± 1.32
18:2		2.0 ± 0.12	2.0 ± 0.12	2.0 ± 0.15	2.0 ± 0.18
20:3n-9		5.9 ± 0.27	5.6 ± 0.34	5.6 ± 0.19	5.5 ± 0.22
20:3n-6		0.5 ± 0.17	0.6 ± 0.03	0.6 ± 0.03	1.0 ± 0.03
20:4		2.7 ± 0.17	2.9 ± 0.15	2.5 ± 0.14	3.8 ± 0.23
22:3n-9		0.9 ± 0.03	0.9 ± 0.15	1.1 ± 0.08	1.1 ± 0.23
20% FBS					
16:0		13.9 ± 0.37	14.4 ± 0.44	16.8 ± 0.29	17.3 ± 0.65
16:1		6.2 ± 0.38	5,5 ± 0.54	6.1 ± 0.29	6.1 ± 0.50
18:0		15.4 ± 0.16	16.3 ± 0.55	14.7 ± 0.38	14.0 ± 0.32
18:1		44.3 ± 0.63	44.1 ± 1.32	42.9 ± 1.29	40.6 ± 0.73
18:2		2.0 ± 0.12	2.0 ± 0.12	2.0 ± 0.15	2.0 ± 0.18
20:3n-9		4.9 ± 0.24	5.4 ± 0.06	5.0 ± 0.12	3.6 ± 0.21
20:3n-6		0.5 ± 0.03	0.7 ± 0.07	0.8 ± 0.03	1.2 ± 0.03
20:4		3.2 ± 0.19	3.6 ± 0.12	3.4 ± 0.22	4.9 ± 0.21
22:3n-9		0.8 ± 0.03	0.9 ± 0.03	0.9 ± 0.12	0.4 ± 0.18
10% Horse serum					
16:0		12.9 ± 0.38	13.9 ± 0.21	15.7 ± 1.14	16.5 ± 0.20
16:1		5.3 ± 0.43	6.0 ± 0.07	4.9 ± 0.29	3.2 ± 0.38
18:0		16.9 ± 0.47	16.7 ± 0.12	16.1 ± 0.18	15.5 ± 0.55
18:1		44.2 ± 1.07	40.5 ± 0.40	38.0 ± 0.66	30.6 ± 0.64
18:2		4.3 ± 0.15	7.3 ± 0.03	9.6 ± 0.36	14.2 ± 0.67
20:3n-9		5.1 ± 0.13	4.7 ± 0.09	4.1 ± 0.37	2.8 ± 0.09
20:3n-6		0.4 ± 0.03	0.4 ± 0.07	1.1 ± 0.09	2.7 ± 0.06
20:4		1.1 ± 0.15	1.2 ± 0.12	1.4 ± 0.21	2.7 ± 0.03
22:3n-9		1.1 ± 0.13	1.2 ± 0.06	1.0 ± 0.0	0.8 ± 0.03

[a]Mean ± SEM (n = 3). [b]nd = Not detected.

TABLE 4
Hemicyst Formation in EFAD–MDCK Cells After 48 Hours of Fatty Acid Supplementation[a]

Supplement	Hemicyst formation (hemicysts/25 low-power fields)
None	3
18:1	5
18:2	157
20:4	73

[a]EFAD–MDCK (essential fatty acid deficient–Madine-Darby canine kidney) cells adapted to growth in medium containing 1% fetal bovine serum plus 19% delipidized serum were grown to confluence in T-25 flasks. This medium was removed and replaced with fresh medium containing no supplemental fatty acid or 10 μM 18:1, 18:2, or 20:4. After 48 h, total hemicysts were counted in five randomly selected low-power fields in each of five flasks, for a total of 25 fields examined.

TABLE 5
Lipid Phosphorous and Cholesterol Content of MDCK Cells Grown in 1, 10, and 20% Fetal Bovine Serum[a]

	Serum concentration of medium (nmol/mg protein)		
Cell lipid	1%	10%	20%
Total lipid phosphorus	225 ± 13	353 ± 15	382 ± 20
Cholesterol	78 ± 5.4	87 ± 4.0	87 ± 3.4
Cholesterol/phospholipid ratio	0.35	0.25	0.23

[a]MDCK cells were grown to confluence in 75 cm^2 flasks in medium containing 1, 10, or 20% serum. Lipid extracts were assayed for inorganic phosphorus and cholesterol as described in the Materials and Methods section. Values are expressed as mean ± SEM (n = 3).

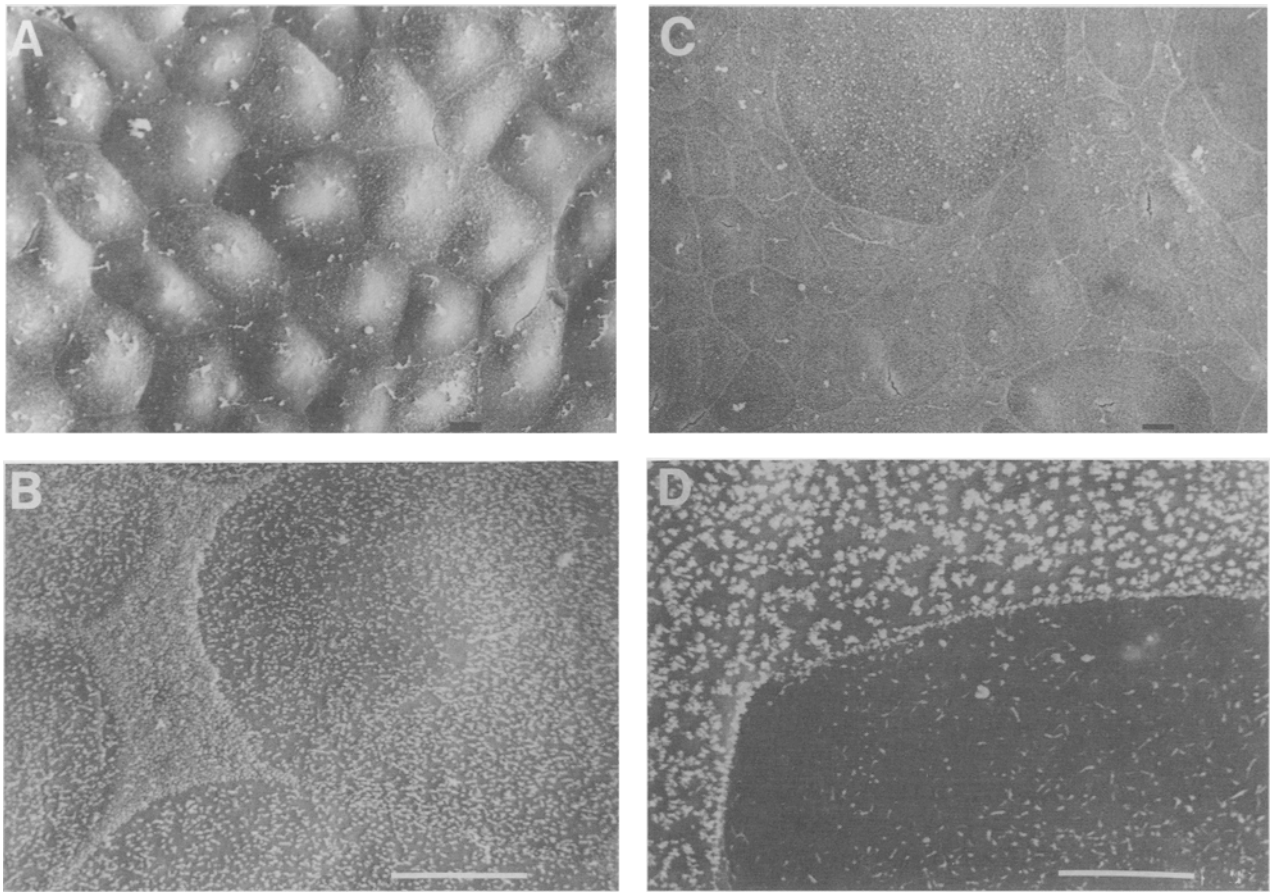


FIG. 4. Scanning electron micrographs (1,680×) of Madin-Darby canine kidney (MDCK) cells grown in 20% fetal bovine serum (FBS) (panels A and B) and essential fatty acid deficient (EFAD)–MDCK cells grown in 1% FBS (panels C and D). Panels A and C, low magnification; Panels B and D, high magnification; bar = 10 μm in all panels.

magnification show clearly that the EFAD–MDCK (C and D) are much flatter than their normal counterparts (A and B), and that individual EFAD–MDCK cells are spread out over a much larger surface area than MDCK cells. SEM also revealed that microvilli are much less dense in EFAD–MDCK than in MDCK, with a 5:1 ratio of MDCK/EFAD–MDCK microvillar density. This ratio was consistent for both the recently divided cells, with relatively sparse microvilli, and for the older cells in the cultures, with greater microvillar densities.

Transmission electron microscopy. Transmission electron microscopy revealed striking differences in the number and distribution of organelles, especially mitochondria. Figure 5 shows low-power micrographs of typical normal MDCK (panel A) and EFAD–MDCK (panels B and C). Panel A, photographed at 2,800×, shows the cytoplasm of the normal MDCK to be densely packed with organelles, which are dispersed more or less evenly, including at the cell periphery (indicated by arrowheads). By contrast, a micrograph of an EFAD–MDCK cell at the same magnification (panel B) shows fewer organelles, and these are for the most part clustered in the perinuclear area, with very few organelles found in the peripheral cytoplasm. Although individual EFAD–MDCK cells extend over a much larger surface area, much of the peripheral cytoplasm (panel C) is amorphous, almost completely lacking in organelles except for free ribosomes and cytoskeletal elements.

Figure 6 is a series of micrographs taken at higher magnification (8,400×) which show additional ultrastructural detail. Panels A and B show the perinuclear area (panel A) and the peripheral cytoplasm (panel B) of normal MDCK cells. These photomicrographs show numerous mitochondria and other organelles, both in the perinuclear area and at the periphery. Panels C and D show cytoplasmic details of EFAD–MDCK at the same magnification; panel C shows the perinuclear area, and panel D part of the peripheral cytoplasm. As seen also in Figure 5, organelles in the EFAD–MDCK are clustered around the nucleus, with much of the peripheral cytoplasm appearing amorphous. The mitochondria in the EFAD–MDCK appear on the average to be larger and more poorly organized than those in the MDCK, with few intact cristae.

DISCUSSION

These results demonstrate that a metabolic state corresponding to EFAD occurs in MDCK renal tubular epithelial cells

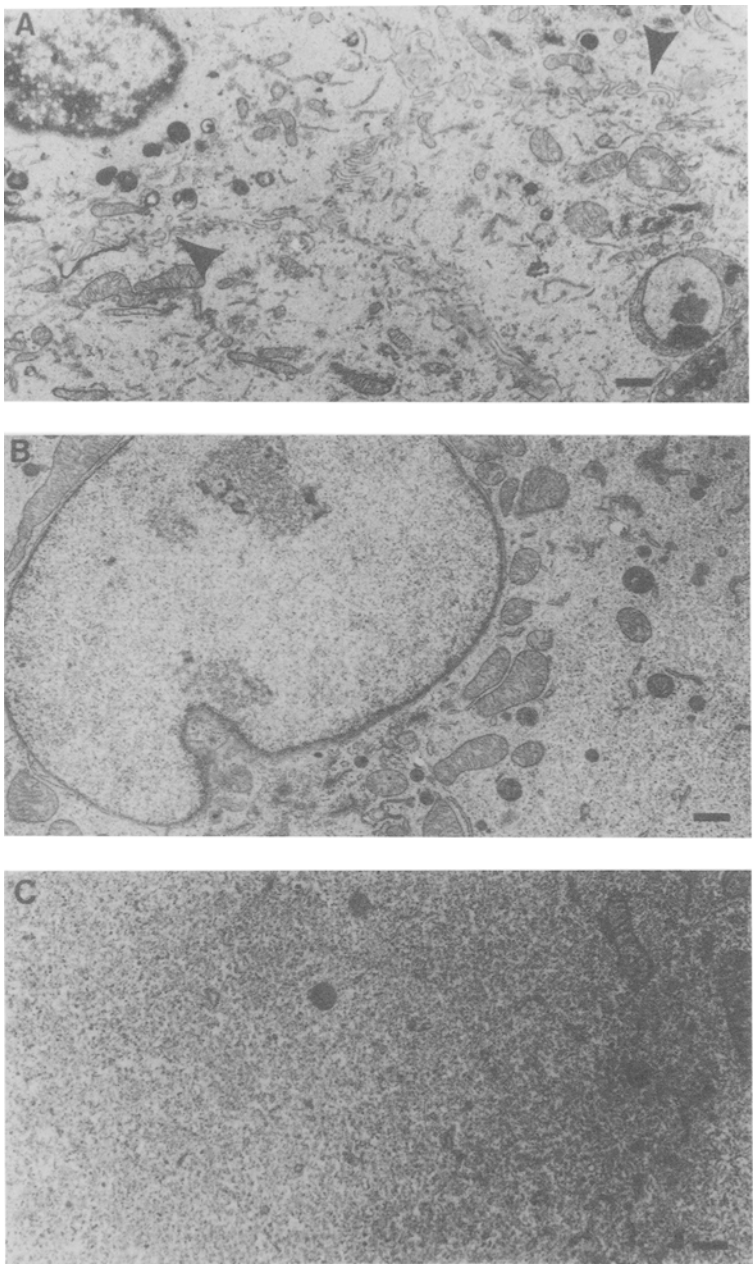


FIG. 5. Transmission electron micrographs (2,800×) of MDCK cells grown in 20% FBS (panel A) and EFAD–MDCK cells (panels B and C). Arrowheads indicate cell–cell boundaries; bar = 1 μm. Abbreviations as in Figure 4.

when they are grown in 1% FBS. This is associated with biochemical changes in phospholipid content and composition, ultrastructural changes as determined by electron microscopy, and functional disturbances as reflected by reduced hemicyst formation. Even when the medium contains 10% FBS, the usual culture condition, fatty acid compositional signs of EFAD are apparent. The finding of enhanced hemicyst formation in MDCK cells grown in medium supplemented with 20% FBS as compared to 10 or 1% supplementation is consistent with an earlier finding that substitution of 10% horse serum for FBS produces enhanced hemicyst formation (24). MDCK cells grown in horse serum, which contains over six times the amount of linoleic acid present in FBS, were found to have 0.8 ± 0.13% 20:3n-9 and 9.0 ± 0.47% 20:4 in their phospholipids (24). Thus MDCK cells grown in 10% horse serum, as opposed to those grown in a similar concentration of FBS, show no signs of EFAD. Hemicyst formation in MDCK cell cultures is an indication of active fluid transport (14,15). This phenomenon is sharply reduced in cells grown under conditions which produce EFAD. As relatively few hemicysts were observed when the medium contained 10% FBS, MDCK cells grown under these ordinary conditions

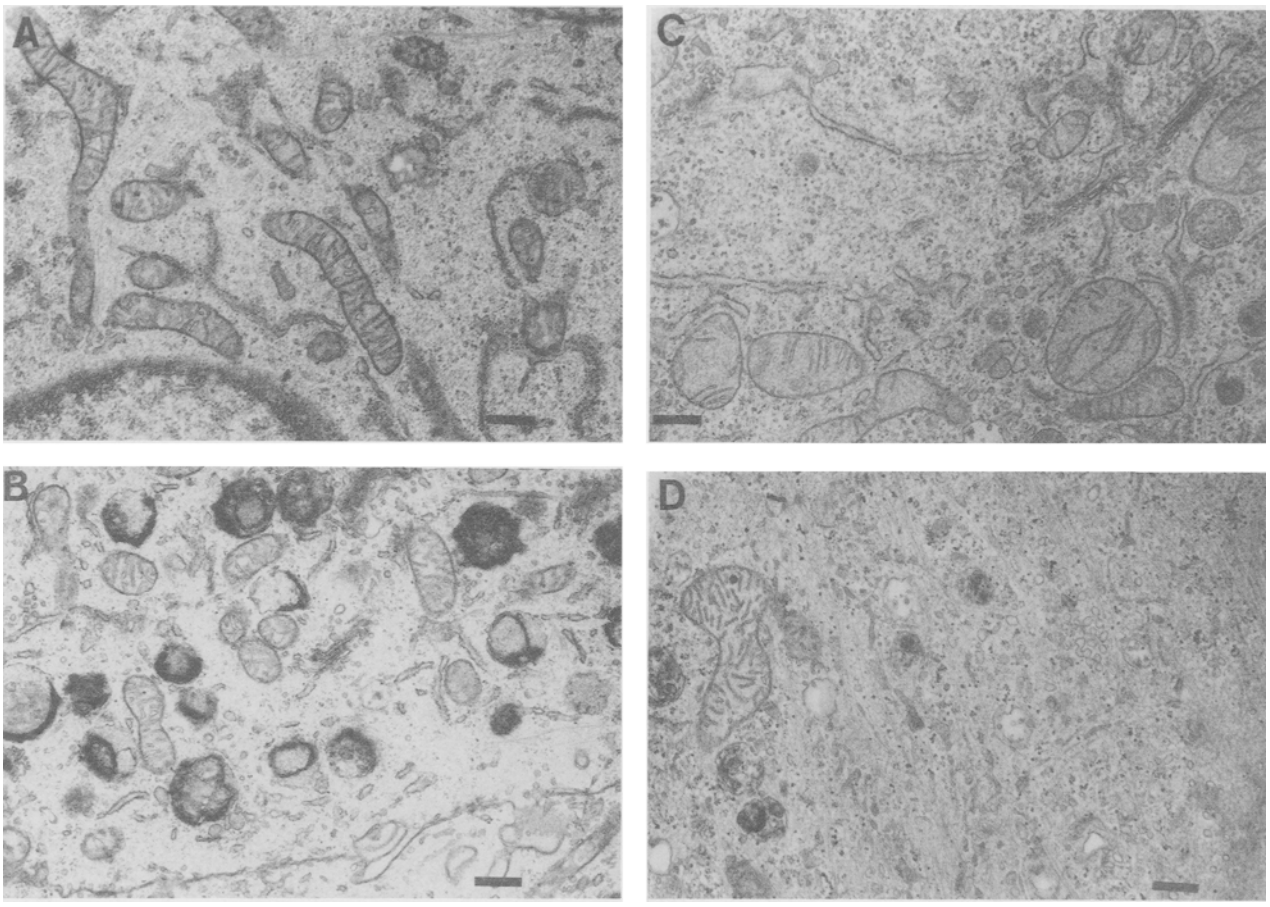


FIG. 6. Transmission electron micrographs (8,400×) of MDCK cells grown in 20% FBS (panels A and B) and EFAD–MDCK grown in 1% FBS (panels C and D). Panels A and C, perinuclear areas; panels B and D, peripheral cytoplasm; bar = 0.5 μm. Abbreviations as in Figure 4.

may not express the full transport potential of normal renal tubular epithelium.

In addition to 20:3n-9, a small amount of a fatty acid likely to be 22:3n-9 accumulates in the EFAD–MDCK lipids. This would be the elongation product of 20:3n-9, and it has been suggested to accumulate in certain cases of EFAD. Consistent with this finding, Dunham *et al.* (25) have reported large amounts of a fatty acid they also believed to be 22:3n-9 in the phospholipids (3.8%) and especially in the triglycerides (23.5%) of renal papillae from rats with long-term (60 wk) EFAD.

The observed ultrastructural differences between the normal MDCK and the EFAD–MDCK can be correlated with the biochemical differences we have found. The total cell lipids of EFAD–MDCK exhibit a large increase in cholesterol/phospholipid ratio compared to those of normal MDCK, caused by a decrease of approximately 50% in EFAD–MDCK cell phospholipids without any accompanying change in cholesterol content (Table 5). Cholesterol is usually found in large amounts in plasma membranes but not in intracellular membranes (26). For this reason, we anticipated that although the EFAD–MDCK plasma membrane probably was relatively unaffected, one or more intracellular membranous organelles whose membranes are composed primarily of phospholipids might be greatly reduced in size or number.

Transmission electron microscopy revealed that the number of such organelles was indeed greatly reduced. This was particularly evident in the peripheral cytoplasm, which was nearly amorphous except for cytoskeletal elements. A deficiency of mitochondria was particularly apparent. Several laboratories have reported mitochondrial dysfunction in studies of EFAD (5,27). This dysfunction has been suggested to result from differences in total number of mitochondria per cell, and possibly also from a functional impairment of these organelles. The present ultrastructural findings support these suggestions. In addition to the reduced numbers of mitochondria, the mitochondria in the EFAD–MDCK appear on the average to be larger and more poorly organized than those in the control MDCK cells, with few unbroken cristae. However, we did not pursue the quantitation of this finding. It is possible that the fragmentary cristae seen in the EFAD cells in Figure 6C is an artifact due to increased membrane fragility during the sectioning process, presumably due to altered membrane lipid composition. However, no such increased breakage is seen in other EFAD membrane structures in the same sections. Such "megamitochondria" also were observed

in rat hepatocytes from animals fed an essential fatty acid-deficient diet (27). Our findings are also consistent with the report by Christon *et al.* (28) of a decreased number of mitochondrial cristae in intestinal mucosal cells of rats fed a diet low in linoleic acid. Similarly, in studies of anterior pituitary phospholipids of rats fed an essential fatty acid-deficient diet, Alessio *et al.* (29) found that 20:4 levels in microsomal plus plasma membrane and nuclear fractions were unchanged compared to those from animals fed control diets, whereas they were decreased in both heavy and light mitochondrial fractions. These authors suggested the existence of a compensatory mechanism which may preferentially allow cells to conserve essential polyunsaturated fatty acids in discrete membrane fractions. It is possible that the reduced phospholipid content of the EFAD–MDCK may limit the capacity of mitochondria to divide. The large numbers of secondary lysosomes observed may also be related to a necessity to conserve membrane components by reprocessing.

The reduction in the density of microvilli on the EFAD–MDCK cell surface also may be related to a decrease in available membrane phospholipid. As the individual EFAD–MDCK have more surface area, there may be no significant reduction in the total number of microvilli per cell. Nevertheless, it seems likely that a cell with a severe fatty acid nutritional deficit might elaborate more, not fewer, microvilli to increase its absorptive surface area if it were not constrained from doing so. Such a phenomenon has been observed in intestinal epithelial cells in diabetic rats, which have a significantly greater surface area than corresponding cells in normal animals (30).

In conclusion, we have characterized an *in vitro* tissue culture model that may be useful for the study of EFAD in the renal distal tubule, using MDCK cells grown in medium supplemented with 1% FBS. The studies reported here show biochemical changes in the cells which are correlated with functional abnormalities and changes in gross and ultrastructural morphology. The abnormalities observed with this system correlate well with published data from animal models of this syndrome in terms of changes in fatty acid composition and transport function in the kidney. Our results also suggest that when MDCK cells are used as models of normal kidney function, the FBS concentration in their growth medium should be increased above the usual 10% level, or some horse serum should be included in the medium to supply additional linoleic acid and, thereby, ensure the presence of an adequate amount of essential fatty acids. Alternatively, supplemental linoleic acid may be added directly to the usual culture medium.

ACKNOWLEDGMENTS

This work was supported in part by Research Grant HL 49,264 from the National Heart, Lung and Blood Institute, National Institutes of Health. Electron microscopy was performed in the University of Iowa Central Electron Microscopy Facility, with the assistance of Dr. Kenneth C. Moore, and gas chromatography/mass spectrometry utilized the University of Iowa College of Medicine GC/MS facility. Technical assistance was provided by Tracy J. Timson.

REFERENCES

1. Lefkowith, J.B., Jakschik, B.A., Stahl, P., and Needleman, P. (1987) Metabolic and Functional Alterations in Macrophages Induced by Essential Fatty Acid Deficiency, *J. Biol. Chem. 262*, 6668–6675.
2. Gavino, G.R., Levy, E., and Gavino, V.C. (1992) Essential Fatty Acid Deficiency Lowers the Activity of the Acetylated Low Density Lipoprotein Receptor of Rat Peritoneal Macrophages, *Biochem. Cell Biol. 70*, 224–227.
3. Levy, E., Garofalo, C., Thibault, L., Dionne, S., Daoust, L., Lepage, G., and Roy, C.C. (1992) Intraluminal and Intracellular Phases of Fat Absorption Are Impaired in Essential Fatty Acid Deficiency, *Am. J. Physiol. 262*, G319–G326.
4. Yokoyama, H., and Matsukawa, R. (1991) Effect of Dietary Essential Fatty Acid Deficiency on $[Ca_i^{2+}]$-ATPase Activity in Microsomal Fraction of Rat Submandibular Gland, *J. Biochem. 23*, 857–861.
5. Gerschenson, L.E., Harary, I., and Mead, J.F. (1967) Studies *in vitro* on Single Beating Rat-Heart Cells. X. The Effect of Linoleic and Palmitic Acids on Beating and Mitochondrial Phosphorylation, *Biochim. Biophys. Acta 131*, 50–58.
6. Holman, R.T. (1987) in *Polyunsaturated Fatty Acids* (Kunau, W.H., and Holman, R.T., eds.) pp. 163–182, American Oil Chemists' Society, Champaign.
7. Lundberg, W.O. (1990) The Significance of *cis, cis, cis* 5,8,11-Eicosatrienoic Acid in Essential Fatty Acid Deficiency, *Nutr. Rev. 38*, 233–235.
8. Cox, J.W., Rutecki, G.W., Francisco, L.L., and Ferris, T.F. (1982) Studies of the Effects of Essential Fatty Acid Deficiency in the Rat, *Circ. Res. 51*, 694–702.
9. Strandvik, B., Brönnegard, M., Gilljam, H., and Carlstedt-Duke, J. (1988) Relation Between Defective Regulation of Arachidonic Acid Release and Symptoms in Cystic Fibrosis, *Scand. J. Gastroenterol. 23*, 1–4.
10. Strandvik, B., Berg, U., Kallner, A., and Kusoffsky, E. (1989) The Effect on Renal Function of Essential Fatty Acid Supplementation in Cystic Fibrosis, *J. Pediatr. 115*, 242–248.
11. Dasgupta, A., Kenny, A.M., and Ahmad, S. (1990) Abnormal Fatty Acid Profile in Chronic Hemodialysis Patients: Possible Deficiency of Essential Fatty Acids, *Clin. Physiol. Biochem. 8*, 238–243.
12. Laposata, M., Prescott, S.M., Bross, T.E., and Majerus, P.W. (1982) Development and Characterization of a Tissue Culture Cell Line with Essential Fatty Acid Deficiency, *Proc. Natl. Acad. Sci. USA 79*, 7654–7658.
13. Laposata, M., Minda, M., Capriotti, A.M., Hartman, E.J., Furth, E.E., and Iozzo, R.V. (1988) Reversible Phenotypic Modulation Induced by Deprivation of Exogenous Essential Fatty Acids, *Lab. Invest. 59*, 838–847.
14. Husted, R.F., Welsh, M.J., and Stokes, J.B. (1986) Variability of Functional Characteristics of MDCK Cells, *Am. J. Physiol. 250*, C214–C221.
15. Rindler, M.J., Chuman, L.M., Shaffer, L., and Saier, M.H. (1979) Retention of Differentiated Properties in an Established Dog Kidney Epithelial Cell Line (MDCK), *J. Cell Biol. 81*, 635–648.
16. Rothblat, G.H., Arbogast, L.Y., Ouellette, L., and Howard, B.V. (1976) Preparation of Delipidized Serum Protein for Use in Cell Culture Systems, *In Vitro 12*, 554–557.
17. Spector, A.A., Kiser, R.E., Denning, G.M., Koh, S.W.M., and DeBault, L.E. (1979) Modification of the Fatty Acid Composition of Cultured Human Fibroblasts, *J. Lipid Res. 20*, 536–547.
18. Fine, J.B., and Sprecher, H. (1982) Unidimensional Thin-Layer Chromatography of Phospholipids on Boric Acid-Impregnated Plates, *J. Lipid Res. 23*, 660–663.
19. Chalvardjian, A., and Rudnicki, E. (1970) Determination of

Lipid Phosphorous in the Nanomolar Range, *Anal. Biochem. 36*, 225–226.
20. Lees, M.B., and Paxman, S. (1972) Modification of the Lowry Procedure for the Analysis of Proteolipid Protein, *Anal. Biochem. 47*, 184–192.
21. U.S. Department of Health and Human Services (1974) *Manual of Laboratory Operations, Lipid Research Clinics Program*, Vol. 1 (NHLBI-NIH 75-628).
22. Hyman, B.T., Stoll, L.L., and Spector, A.A. (1981) Accumulation of (n-9)-Eicosatrienoic Acid in Confluent 3T3-L1 and 3T3 Cells, *J. Biol. Chem. 256*, 8863–8866.
23. Taub, M., Chuman, L., Saier, M.H., and Sato, G. (1979) Growth of Madin–Darby Canine Kidney Epithelial Cell (MDCK) Line in Hormone-Supplemented, Serum-Free Medium, *Proc. Natl. Acad. Sci. USA 76*, 3338–3342.
24. Stoll, L.L., and Spector, A.A. (1984) Changes in Serum Influence the Fatty Acid Composition of Established Cell Lines, *In Vitro 20*, 732–738.
25. Dunham, E.W., Balasingham, M., Privett, O.S., and Nickell, E.C. (1978) Effects of Essential Fatty Acid Deficiency on Prostaglandin Synthesis and Fatty Acid Composition in Rat Renal Medulla, *Lipids 13*, 892–897.
26. Bretscher, M.S. (1973) Membrane Structure: Some General Principles, *Science 181*, 622–629.
27. Kryvi, H., Christiansen, E., Tangeras, A., and Flatmark, T. (1986) Effects of Essential Fatty Acid Deficiency on Mitochondria and Peroxisomes in Rat Hepatocytes with Special Reference to a Partially Hydrogenated Fish Oil Diet, *Biochim. Biophys. Acta 881*, 87–92.
28. Christon, R., Meslin, J.C., Thévenoux, T., Linard, A., Léger, C.L., and Delpal, S. (1991) Effects of a Low Dietary Linoleic Acid Level on Intestinal Morphology and Enterocyte Brush Border Membrane Lipid Composition, *Reprod. Nutr. Dev. 31*, 691–701.
29. Alessio, M.L., Wandscheer, D.E., Soares, M.C., Clauser, H., Enjalbert, A., Kordon, C., and Léger, C.L. (1992) Effect of an Essential Fatty Acid Deficiency on the Phospholipid Composition in Anterior Pituitary Membranes, *Biochem. Biophys. Res. Commun. 183*, 1047–1055.
30. Miller, D.L., Hanson, W., Schedl, H.P., and Osborne, J.W. (1977) Proliferation Rate and Transit Time of Mucosal Cells in Small Intestine of the Diabetic Rat, *Gastroenterology 73*, 1326–1332.

[Received January 14, 1995, and in final revised form September 26, 1995; Revision accepted October 18, 1995]

The Essential Oil from *Tagetes minuta* L. Modulates the Binding of [^{3}H]Flunitrazepam to Crude Membranes from Chick Brain

Daniel A. García[a], María A. Perillo[a,*], Julio A. Zygadlo[b], and Irene D. Martijena[a]

[a]Cátedra de Química Biológica and [b]Cátedra de Química Orgánica, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, 5000-Córdoba, Argentina

ABSTRACT: The hypothesis that the essential oil from *Tagetes minuta* L. can interact with biological membranes was investigated by assessing its ability of perturbing the binding of a benzodiazepine [flunitrazepam (FNTZ)] to crude membranes from chick brains. The essential oil from *T. minuta* L. inhibited [^{3}H]FNTZ specific binding to chick brain membranes. These values were obtained from the analysis of the saturation curve for the kinetic parameters: dissociation equilibrium constant (K_d) = 2.47 ± 0.32 nM, maximal binding (B_{max}) = 556 ± 5 fmoles/mg protein, and Hill coefficient (n) = 1.00 ± 0.07 in the absence, and K_d = 6.73 ± 1.4 nM, B_{max} = 583 ± 69 fmoles/mg protein, and n = 1.02 ± 0.08 in the presence of 29 μg/mL of essential oil. The essential oil could self-aggregate with a critical micellar concentration (CMC) of 60 μg/mL. The marked increase in [^{3}H]FNTZ nonspecific binding starting at 60 μg of essence per mL was due to that phenomenon and revealed the ability of self-aggregated structures to interact with membranes. [^{3}H]FNTZ specific binding decrement as a function of essence concentration cannot be ascribed merely to oil's micelles ability of trapping the lipophilic radioligand molecules, because the discontinuous behavior that characterizes a monomer-aggregate phase transition was not shown. Oil's components might behave as competitive inhibitors or allosteric modulators of FNTZ specific binding. However, their ability to increase FNTZ nonspecific binding at concentrations below oil's CMC suggests that this effect may be due to oil's partitioning into the lipid bilayer. This latter phenomenon would induce an increment in membrane fluidity and a change on FNTZ binding site toward a lower affinity conformation. Therefore, the essential oil components can interact with brain membranes either as monomers, by partitioning into the lipid bilayer, or as self-aggregated structures, through an adsorption to the membrane surface.
Lipids 30, 1105–1110 (1995).

Tagetes minuta L. is a plant widely spread over the Argentinean territory. The composition of *T. minuta* L. essential oil has been described (1,2). Most of its components are terpene derivatives. Furthermore, some terpenes have been previously shown to affect membrane fluidity (3), and other terpenes behaved as γ-aminobutyric acid $(GABA)_A$ receptor complex inhibitors (4); the present essential oil could inhibit the development of fungus in culture through a mechanism that may involve instabilization of biological membranes (5).

Modifications in membrane organization can be transduced as changes in ionic permeabilities, in activities of membrane enzymes, in affinities of membrane receptors, etc. These could be due to changes in proteins' conformations exerted either directly, through the binding of a specific ligand, or indirectly, through phase transitions within lipidic domains (6,7).

$GABA_A$ is a supramolecular complex of several integral membrane proteins that includes three major binding sites—one for GABA, one for 1,4-benzodiazepines, and a third site for picrotoxine and barbiturates. Those sites are linked so that the binding of one of those ligands to its specific site can allosterically modulate the binding of another ligand to its own receptor (8–10). In addition, the sensitivity of the $GABA_A$-complex may fluctuate because of conformational constraints imposed by the lipid microenvironment (11).

As a lipophilic substance, the benzodiazepines (BZD) flunitrazepam (FNTZ) can partition into the hydrophobic domains of the membrane through nonspecific interactions (12) and increase membrane fluidity (13), as well as binding specifically to its receptor site at the $GABA_A$ receptor complex. Conversely, changes in membrane organization can modify FNTZ partition coefficient, as proven by the differences in FNTZ partition coefficient in model membranes above and below lipid phase transition temperature (García, D.A., and Perillo, M.A., unpublished data).

As both specific and nonspecific bindings of FNTZ were modulated by changes in membrane organization, it appeared that FNTZ would be a sensitive molecular probe for testing the ability of the essential oil to interact with biological membranes. Moreover, we also considered the possibility that not only the nonspecific, but also the specific, FNTZ binding were affected by the essence. Then, it would be justified to think of other possible effects of the essence, beyond the antifungic, such as its possible ability to be an antifeedant in insects, as well as an anxiolytic and/or anticonvulsant in vertebrates.

*To whom correspondence should be addressed at Cátedra de Química Biológica, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Velez Sarsfield 299, 5000 Córdoba, Argentina.

Abbreviations: B_{max}, maximal binding; BZD, benzodiazepine; CMC, critical micellar concentration; DZ, diazepam; FNTZ, flunitrazepam; GABA, γ-aminobutyric acid; GC, gas chromatography; K_d, dissociation equilibrium constant.

Copyright © 1995 by AOCS Press

In the present paper, we assessed the ability of *T. minuta* essential oil of perturbing the *in vitro* binding of a BZD to crude membranes from chick brain.

MATERIALS AND METHODS

BZD [diazepam (DZ) and FNTZ] were obtained from Hoffman La Roche (Basle, Switzerland). [^{3}H]FNTZ was purchased from New England Nuclear Chemistry (E.I. DuPont de Nemours & Co., Inc., Boston, MA). Other drugs and solvents were of analytical grade. Plant material consisted of aerial parts of *T. minuta* L. collected in March from plants growing in the locality of Sesenta Curvas, Córdoba, Argentina. Voucher specimens have been deposited in the Botanical Garden Museum of Córdoba (herbarium CORD 79).

Extraction and characterization of the essential oil from T. minuta *L.* The oil was isolated from fresh plant material by hydrodistillation for three hours, using a homemade Clevenger-type apparatus (1,2,14). The very essential oil was used as the sample for gas chromatography (GC), and the ethyl acetate extract of an oil–water partition was used to find the composition of water-solubilized components of the essence. GC analysis was performed using a Shimadzu (Tokyo, Japan) GC-GA gas chromatograph equipped with a flame-ionization detector, a data handling system, and a fused-silica column (25 × 0.25 mm i.d.). Oven temperature was programmed from 70 to 200°C at a scanning rate of 40°C/min; the injector and detector were set at 280°C; the carrier gas was helium at a flow rate of 2 mL/min. The percent composition of the oil was computed from the GC peak areas (1,2). The GC/mass spectrometry unit consisted of a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph equipped with an SE54 fused silica column (25 × 0.25 mm i.d.). The chromatograph was coupled to an HP5971A mass selective detector at 70 eV. The identity of the components was assigned by comparison of their retention indices and mass spectra with corresponding data of components of reference oils (1,2,14).

Effect of T. minuta *essential oil on the interaction of [^{3}H]flunitrazepam with brain membranes: incubation system.* The membrane receptor preparation was obtained as follows: whole brains from recently sacrificed chicks were dissected on ice and homogenized with 50 mM Tris–HCl pH 7.4 cold buffer (50 mL per gram of wet tissue); this suspension was centrifuged at 36,000 × *g* for 20 min at 40°C. The pellet was stored at −20°C, and it was resuspended in the same buffer immediately before use.

The incubation system contained (in 650 µL final volume) the membrane suspension at a final concentration of 1 mg protein/mL, Tris–HCL buffer containing (nonspecific) or not (total) DZ 9.2 µM (final concentration) and [^{3}H]FNTZ (specific activity 87 Ci/mmol) to reach final concentrations ranging from 0.7 to 16 nM (saturation curve) or a fixed concentration of 1 or 2 nM (other experiments). The essential oil from *T. minuta* L. was incorporated in 10 µL of an aqueous dispersion of a proper concentration. The samples were incubated at 4°C in the dark for 1 h, and then 200-µL aliquots were added to 2 mL of cold Tris–HCl buffer and immediately filtered through Whatman (Maidstone, England) GF/B filters. Those filters were rinsed with another 2 mL of cold buffer, dried in air, and placed in glass vials containing 2.5 mL of scintillation cocktail (Triton 30%, diphenyloxazole 0.3% in toluene); radioactivity was determined with a precision of 5% and a counting efficiency of 60% for tritium in a Rackbeta LKB 1224 scintillation spectrometer (Pharmacia Wallac OY, Turku, Finland). The whole filtration procedure was completed in 10 s. The filters used had been treated for 4 h at 4°C with isobutanol 4% (vol/vol) in water to minimize their capacity of adsorbing BZD. Protein concentrations were determined by a modified Lowry procedure (15).

Determination of the critical micellar concentration (CMC). Variable amounts of the essential oil (from 0 to 2.3 mg) were dispersed in 1 mL of water at room temperature; 10 µL of 3.3% (wt/vol) thymol blue methanolic solution were added, and the absorbance was measured at 720 nm with a Spectronic 21 spectrophotometer (Bausch & Lomb Inc., Rochester, NY). The CMC was determined as the concentration value corresponding to the interception with the abscissa axis of the asymptote to the curve of absorbance at 720 nm vs. essential oil concentration.

Statistical analysis. The saturation curves were adjusted to the following equation (16):

$$B = \frac{B_{max} \cdot F}{K_d + F} \qquad [1]$$

by a nonlinear regression analysis performed by a computed added least squares method (B and F were the bound and free [^{3}H]FNTZ concentrations, respectively, B_{max} = maximal binding, K_d = equilibrium dissociation constant). Comparisons among means were performed by a two-tailed Student's t-test; the propagation of errors was applied to compute the standard deviation of magnitudes calculated from two or more experimentally determined parameters (17).

RESULTS

The *T. minuta* L. essential oil was a complex mixture of mostly hydrophobic substances. Its density was 0.94 g/mL. Table 1 shows the composition of the essential oil and also that of its aqueous dispersion. The *cis* isomer of ocimene is the major component of the essential oil, representing 51.32% by weight of the total essence. A group of compounds that includes *t*-ocimene, dihydrotagetone, *c*-tagetone, *c*-ocimenone, *t*-ocimenone, and spathulenol represent 33.74%, with individual abundances ranging between 2 and 7.8%; fifteen other compounds with abundances of 1.48% or lower add 7% and, finally, 7.95% that corresponds to 20 chromatographic peaks remained unidentified. The highest concentrated compounds in the essential oil, although in different proportions, were also the major components found in the water phase. As expected, the relative proportion of hydrocarbon-type compounds decreased and that of the aldehydes, cetones, alco-

TABLE 1
Chemical Composition of the Essential Oil from *Tagetes minuta* L.[a]

Compound	Total essence[a] (percentage)	Water-soluble[b] (percentage)	Water-soluble/total
Hydrocarbons			
β-Pinene	1.48	0.20	0.13
α-Pinene	1.03	0.18	0.17
c-Ocimene	51.32	11.07	0.22
t-Ocimene	7.80	1.78	0.23
Limonene	0.23	0.00	0.00
γ-Terpinene	0.43	0.00	0.00
Terpinolene	0.12	0.00	0.00
α-Thujene	0.08	0.00	0.00
α-Copaene	0.10	0.00	0.00
γ-Elemene	0.07	0.00	0.00
Aldehydes and cetones			
Dihydrotagetone	7.73	14.61	1.89
c-Tagetone	4.17	8.07	1.93
t-Tagetone	0.13	0.69	5.30
c-Ocimenone	6.17	18.46	2.99
t-Ocimenone	5.78	18.48	3.20
Carvona	0.88	1.71	1.93
Dihydrocarvone	0.91	1.86	2.04
Alcohols and oxides			
Linalool	0.08	0.24	3.00
Limonene oxide	0.33	3.18	9.64
Borneol	0.65	1.03	1.59
Carveol	0.85	3.03	3.56
Spathulenol	2.09	7.46	3.57
Nonidentified	7.95	7.58	0.95

[a,b]The oil composition was determined by gas chromatography/mass spectrometry. Pinene: bicycle [3.1.1]hept-2-ene-2,6,6-trimethyl; ocimene, 1,3,6-octatriene-3,7,dimethyl; limonene, cyclohexene-1-methyl-4-(1-methylethenyl)-(R); terpinene, 1,3-cyclohexadiene-1-(methyl)-4-(1-methylethyl); terpinolene, cyclohexen-1-methyl-4-(1-methylethylidene); α-thujene, bicycle [3.1.0] hex-2-ene-2-methyl-5-(1-methylethyl)-(1R); α-copaene, tricycle [4.4.0.$0^{2,7}$]dec-3-ene-1,3-dimethyl-8-(1-methylethyl); *t*-elemene, cyclohexene-6-ethenyl-6-methyl-1-(1-methylethyl)-3-(1-methylethylidene)-(S); dihydrotagetone, 7-octen-4-one-2,6-dimethyl; tagetone, 5,7-octadien-4-one-2,6-dimethyl-(E); ocimenome, 2,5,7-octatrien-4-one-2,6-dimethyl; carvona, 2-cyclohexen-1-one-2-methyl-5-(1-methylethenyl)-(R); dihydrocarvona, cyclohexanone-2-methyl-5-(1-methylethenyl); linalool, 1,6-octadien-3-ol-3,7-dimethyl; limonene oxide, 1,2-epoxy-cyclohexene-1-methyl-4-(1-methylethenyl)-(R); borneol, bicycle-[2,2,1]-heptan-2-ol-1,7,7-trimethyl-(1S-Endo); carveol, 2-cyclohexen-1-ol-2-methyl-5-(1-methylethenyl); spathulenol, ^{1}H-cycloprop[e]azulen-7-ol-decahydro-1,1,7-trimethyl-4-methylene-1aR-(1aα,4aα-7aβ,7aα)].

hols, and oxides increased in the water phase compared with that of the total essential oil.

As shown in Table 2, in the presence of 29 μg/mL of *T. minuta* essential oil the specific binding of 1 nM [^{3}H]FNTZ to its receptor sites at chick brain membranes decreased significantly with respect to the control. However, the nonspecific binding increased as a function of the final oil concentration.

The absorbance at 720 nm (A_{720}) of thymol blue (Fig. 1a) and the nonspecific binding (Fig. 1b) were constant up to 60 μg of essence per mL of aqueous solution. Beyond this point, both parameters rose abruptly as a function of the essence concentration. Identical results were obtained when mixtures of [^{3}H]FNTZ with variable amounts of essential oil were filtrated through glass filters that had previously retained the same membrane suspension used in the nonspecific test tubes; however, with membrane-free filters, no radioactivity was recovered along the same range of essence concentrations (Fig. 1b). The specific binding of [^{3}H]FNTZ decreased as a function of the essence concentration (Fig. 1c), but, instead of what occurred with the parameters measured in Figure 1a and 1b, which showed discontinuous behavior, this decrement increased continuously, starting from the lowest essence concentration assayed.

Figure 2a shows that the specific binding of [^{3}H]FNTZ to membrane receptors increased as a function of free [^{3}H]FNTZ concentration in the incubation system, reaching a plateau at approximately 8 nM. In the presence of 29 μg of *T. minuta* essential oil, an inhibition of the binding was observed mainly at low [^{3}H]FNTZ; here, a plateau was reached at a radioligand concentration higher than in the absence of essence. From the nonlinear regression analysis of the saturation curves, the following values were determined for the binding parameters: $K_d = 2.47 \pm 0.32$ nM and $B_{max} = 556 \pm 5$ fmoles/mg protein, and $K_{d,e} = 6.73 \pm 1.4$ nM and $B_{max,e} = 583 \pm 69$ fmoles/mg protein in the absence and in the presence of the essential oil, respectively. The difference between K_d and

TABLE 2
Binding of [^{3}H]FNTZ to Chick Brain Membranes as a Function of *Tagetes minuta* L. Essential Oil[a]

Essential oil concentration (μg/mL)	Binding of [^{3}H]FNTZ (fmol/mg protein)		
	Total	Nonspecific	Specific
0	110.3 ± 5.0	6.5 ± 0.5	103.5 ± 5.5
29	82.8 ± 3.4*	6.2 ± 0.3	76.6 ± 3.7*
145	95.4 ± 2.1*	12.3 ± 1.2*	83.1 ± 3.3*
273	102.1 ± 1.2	27.5 ± 1.4*	74.6 ± 2.6*

[a]The binding of 1 nM [^{3}H]flunitrazepam (FNTZ) to chick brain membranes was measured in the presence (nonspecific) or in the absence (total) of nonlabeled diazepam. Specific: results from the difference between total minus nonspecific. The experimental details are explained in the Materials and Methods section. The values shown correspond to the means of triplicate determination ± SEM; *significatively different ($P < 0.05$) with respect to the control (without essential oil).

$K_{d,e}$ was significant from the statistical point of view ($P < 0.01$). B_{max} and $B_{max,e}$ were not statistically different ($P > 0.1$). The Scatchard plot of the saturation curve obtained without the essential oil was linear, as described previously (18); the presence of the oil did not change this characteristic (Fig. 2a, inset). Hill coefficients were equal to unity in the presence (1.02 ± 0.08) and in the absence (1.00 ± 0.07) of the essential oil (not shown).

In the absence and in the presence (below CMC) of the essence, the nonspecific binding increased as a function of [^{3}H]FNTZ concentration (Fig. lb); in both cases the points were adjusted to straight lines passing through the origin, but the slope in the presence of essence was higher than in the absence of the essence (7.14 and 4.24 fmol/mg prot/nM, respectively).

DISCUSSION

The essential oil from *T. minuta* L. could inhibit the specific binding of [^{3}H]FNTZ to chick brain membranes (Fig. 1c, Fig. 2a, Table 2). Whether this effect was actually exerted at the membrane level was the question that arose.

The increase of A_{720} of thymol blue as a function of the essential oil concentration in an aqueous solution (Fig. 1a) could be interpreted on the basis of the spontaneous self-aggregation of the essence component molecules after a critical point of essence concentration in the aqueous phase was overcome (CMC). These self-aggregated structures constitute a new hydrophobic phase in the system where the dye, also hydrophobic, is more soluble than in water. This phenomenon was made evident by the abrupt increase of the dye absorbance beyond an essence concentration of 60 μg/mL. As BZD is also a high lipophilic substance, it could be preferentially partitioned into the mentioned structures as well. This mechanism can explain the discontinuous increment of [^{3}H]FNTZ nonspecific binding (Fig. lb). On the other hand, these structures are not big enough to be retained by clean glass filters, as suggested by the flatness of the plot of radioactivity vs. oil concentration (Fig. lb). However, the fact that these self-aggregated structures were retained by membrane-coated filters following a radioactivity pattern similar to that of nonspecific binding (Fig. lb) points out their high tendency to establish surface–surface interaction with biological membranes.

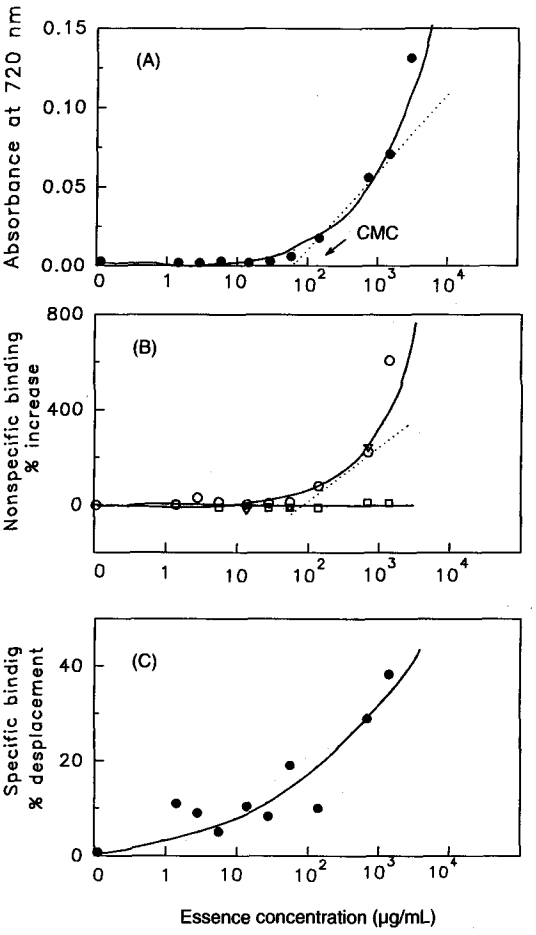


FIG. 1. Effect of *Tagetes minuta* L. essential oil concentration on the self-aggregation of its components and on the specific and nonspecific interaction of [^{3}H]flunitrazepam (FNTZ) with chick brain membranes. (A) Variation of thymol blue absorbance at 720 nm, CMC, critical micellar concentration; (B) ○, nonspecific binding of [^{3}H]FNTZ (2 nM) to chick brain membranes (the displacement agent was 10 μM diazepam); ▽, mixture of essential oil plus 2 nM [^{3}H]FNTZ filtrated through glass filters which had previously retained the same membrane suspension used in the nonspecific test tubes; □, mixtures of essential oil plus 2 nM [^{3}H]FNTZ filtered through membrane-free glass filters. (C) displacement percentages of [^{3}H]FNTZ (2 nM final concentration) specific binding with respect to the control without essence. Values are the mean ± SEM of triplicate determination; the values in (C) come from four experiments, each one in triplicate.

The specific binding of [^{3}H]FNTZ decreased as a function of the essence concentration (Table 2, Fig. 1c), and, contrary to what happened with the A_{720} of thymol blue and the nonspecific binding of [^{3}H]FNTZ, it did not show the discontinuous behavior typical of a phase transition process. In fact, the specific binding decreased continuously, starting from the lowest concentration tested (Fig. 1c).

The presence of self-aggregated structures might decrease the ligand effective concentration in the aqueous phase; consequently, this would induce an artificial decrement of the

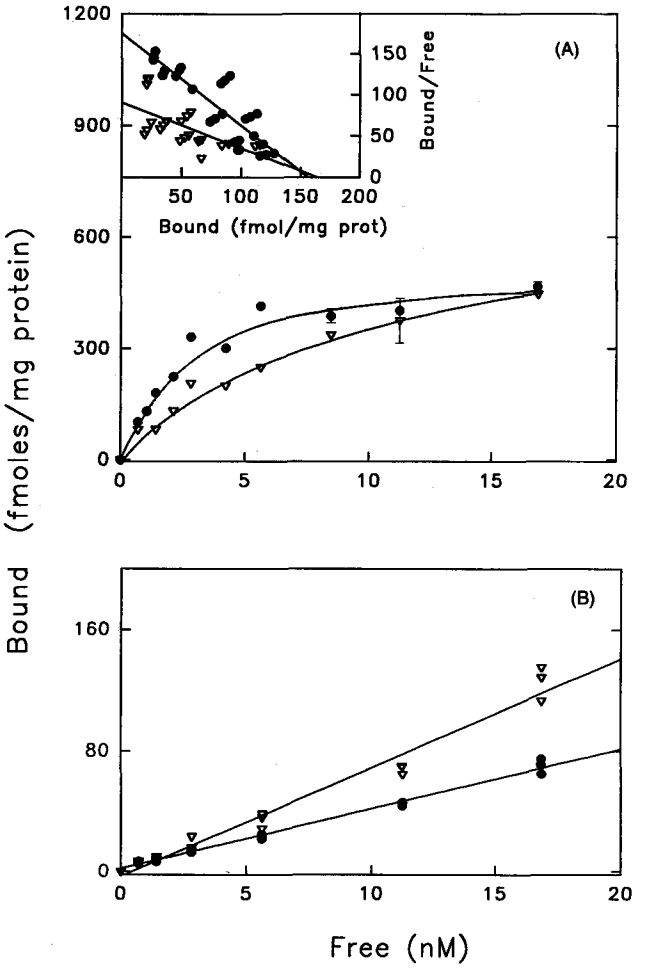


FIG. 2. Binding of [^{3}H]FNTZ to chick brain membranes as a function of [^{3}H]FNTZ concentration. (A) Saturation curves of the specific binding (total binding minus nonspecific binding) of [^{3}H]FNTZ; Inset: Scatchard plots of the saturation curves; (B) nonspecific binding of [^{3}H]FNTZ as a function of total [^{3}H]FNTZ concentration in the absence (●) or in the presence (▽) of 29 µg of essential oil from *Tagetes minuta* L. per mL of incubation system. Nonlabelled diazepam, 10 µM, was used in the nonspecific test tubes as the displacement agent. Points are the mean of three determinations; error bars represent the SEM values. Abbreviation as in Figure 1.

ligand specific binding that could be erroneously interpreted as true inhibition of the bindings This decrease in the specific binding, which would be higher at low ligand concentrations, could be overcome at higher ligand concentrations and might be misinterpreted as a competitive inhibition. However, this mechanism can only explain the decrement in specific binding observed over the essence CMC, but not the one observed below this concentration value.

From the regression analysis of the saturation curves shown in Figure 2a, it was found that, in the presence of 29 µg/mL of the essential oil, the affinity of the ligand binding decreased while the maximal binding remained constant, compared with the values obtained with control samples without essence. Because the essential oil concentration used in this experiment was well below the essence CMC value, the phenomenon of ligand trapping could not take place in the system. Therefore, that decrease in K_d cannot be ascribed to a mere artificial decrement in the free ligand concentration; on the contrary, it can be considered either a displacement of ligand from the receptor site at the membrane or an allosteric effect leading to a decrement of ligand-binding affinity.

If a competitive inhibitory mechanism takes place, the best candidates for the competitive agent may be found among the aldehydes and ketones because these were the essence components that were more concentrated in the aqueous phase (Table 1). In addition, these would probably be the molecules more capable of leaving any self-aggregated structure, establishing multiple chemical equilibria micelle ↔ water ↔ membrane, well displaced toward the right. On the other hand, the putative inhibitory agent(s) did not discriminate among the different BZD-receptor subtypes, as the Hill coefficient was not different from the unity in the presence of the essential oil (16,18). However, although not made evident by the saturation curve shape, an allosteric effect cannot be discarded, as the antagonistic effects on the $GABA_A$ receptor complex exerted by terpenoid epoxides have already been proven (4).

Much experimental evidence pointed out that changes in the membrane lipid microenvironment (19,20,21) play an important role in regulating ligand binding to BZD receptors. For that reason, oil-induced modifications in the general membrane properties that can cause a conformational change in one or more protomers of the $GABA_A$ complex and lead to a decrement in the affinity of the BZD receptor for FNTZ should also be considered.

The ability of oil's self-aggregated structures to interact with membranes has been demonstrated in this paper by the increment, as a function of oil concentration, of the radioactivity retained upon the filtration of [^{3}H]FNTZ–oil mixtures through membrane-coated filters only after an oil concentration threshold in the mixture (equivalent to oil's CMC) had been passed over (Fig. 1b). Moreover, the increment in [^{3}H]FNTZ nonspecific binding in the presence of essence concentrations lower than its CMC (nonassociated), with respect to essence-free samples, suggested the possibility that oil components could have partitioned into the lipid bilayer, increasing membrane fluidity. This conclusion is supported by the results we obtained with artificial membranes; we showed that the partitioning of FNTZ into the membrane from the aqueous media is favored by a lipid phase transition from a highly organized structure, the gel phase, to a less organized arrangement, the liquid crystalline phase (Perillo, M.A., and García, D.A., unpublished data).

An enhancement of [^{3}H]DZ binding by nonsaturated fatty acids was observed *in vitro* in membranes of most animal species, although an inhibition was shown in some fish species (21); it was claimed that this effect would not be merely a change in membrane fluidity. However, no comments had been made in relation to the behavior of nonspecific binding, and/or about the possible relationship among the effects observed and the self-aggregating properties of the

perturbing agents that (as shown here) may induce artificial effects in some conditions. For that reason, we believe that the approach used in the present paper may give new insights into the way some binding phenomena should he analyzed.

In conclusion, the essential oil from *T. minuta* can interact with biological membranes as demonstrated by its ability to perturb both [^{3}H]FNTZ specific binding (decrement in K_d at a concentration lower than oil's CMC) and [^{3}H]FNTZ non-specific binding (increment in [^{3}H]FNTZ partitioning at oil's concentration below CMC and, above oil's CMC, increment of the adsorption to the membrane of [^{3}H]FNTZ-containing oil's self-aggregated structures). Thus, essential oil-induced changes in membrane dynamics may explain oil's biological effects on fungus. The inhibitory effects demonstrated *"in vitro"* had a pharmacological correlate *"in vivo,"* as the oil exerted anxiogenic actions on rats (Martijena, I.D., García, D.A., Marín, R.H., Zygadlo, J.A., Molina, V.A., and Perillo, M.A., unpublished data). Experiments to investigate *in vitro* and *in vivo* effects of the individual major components of this essential oil are being carried out in order for their activities to be compared with that of the whole essence. Additivity, synergism, or antagonism among the activities of the different components could be demonstrated if the mixture equals, is more, or is less active, respectively, than either pure compound.

ACKNOWLEDGMENTS

This paper was supported by grants from CONICOR and SECYT-Universidad Nacional de Córdoba, Córdoba, Argentina.

REFERENCES

1. Zygadlo, J.A., Grosso, N.R., Abburra, R.E., and Guzman, C.A. (1990) Essential Oil Variation in *Tagetes minuta* Populations, *Biochem. Syst. Ecol. 18,* 405–407.
2. Zygadlo, J.A., Lamarque, A.L., Maestri, D.M., Guzman, C.A., and Grosso, N.R. (1993) Composition of the Inflorescence Oils of Some Tagetes Species from Argentina, *J. Essen. Oil Res. 5,* 679–681.
3. Bard, M., Albrecht, M.R., Gupta, N., Gynn, C.J., and Stillwell, W. (1988) Geraniol Interferes with Membrane Functions in Strains of Candida and Saccharomyces, *Lipids 23,* 534–538.
4. Mullin, C.A., Alfatafta, A.A., Harman, J.L., Everett, S.L., and Serino, A.A. (1991) Feeding and Toxic Effects of Floral Sesquiterpene Lactones, Diterpenes and Phenolics from Sunflowers (*Heliantus annus* L.) on Western Corn Rootworm, *J. Agric. Food Chem. 39,* 2293–2299.
5. Zygadlo, J.A., Guzman, C.A., and Grosso, N.R. (1994) Antifungal Properties of the Leaf Oils of *Tagetes minuta* L and *Tagetes filifolia Lag., J. Essent. Oil Res. 6,* 617–621.
6. Berridge, M.J. (1987) Inositol Trisphosphate and Diacylglycerol: Two Interacting Second Messengers, *Annu. Rev. Biochem. 56,* 159–193.
7. Siegel, D.P. (1987) Membrane-Membrane Interactions *via* Intermediate in Lamellar-to-Inverted Hexagonal Phase Transitions, in *Cell Fusion* (Sowers, A.E., ed.) Plenum Press, New York, pp. 81–207.
8. Haefely, W.E. (1989) Pharmacology of the Benzodiazepine Receptor, *Eur. Arch. Psychiatr. Neurol. Sci. 238(5–6),* 294–301.
9. Martin, I.L. (1987) The Benzodiazepines and Their Receptors: 25 Years of Progress, *Neuropharmacology 26,* 957–970.
10. Stephenson, F.A. (1988) Understanding the GABAA Receptor: A Chemically Gated Ion Channel, *Biochem. J. 249,* 21–32.
11. Guidotti, A., Corda, M.G., Wise, B.C., Vaccarino, F., and Costa, E. (1983) GABAergic Synapses. Supramolecular Organization and Biochemical Regulation, *Neuropharmacology 22(12B),* 1471–1479.
12. Perillo, M.A., and Arce, A. (1991) Determination of the Membrane-Buffer Partition Coefficient of Flunitrazepam, A Lipophilic Drug, *J. Neurosci. Methods 31,* 203–208.
13. Kurishingal, H., Barain, P., and Restall, C. (1992) Benzodiazepine-Induced Changes in Biomembrane Fluidity, *Biochem. Soc. Trans. 20,* 1575.
14. Lawrence, B.M., Powel, R.H., Smith, T.W., and Kramer, S. (1986) Essential Oils of the Tagetes genus, *Perf. and Flav. 10,* 56–58.
15. Markwell, M.A.K., Haas, S., Bieber, L.L., and Tolbert, N.E. (1978) A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples, *Anal. Chem. 87,* 206–210.
16. Bennett, J.P. Jr. (1978) Methods in Binding Studies, in *Neurotransmitter Receptor Binding* (Yammamurga, H.I., Enna, S.J., and Kuhar, J.K., eds.) Raven Press, New York, pp. 57–90.
17. Green, J.R., and Margerison, D. (1978) *Statistical Treatment of Experimental Data,* Chapter 15, Elsevier, New York, pp. 320–329.
18. Burt, D.R., and Kamatchi, G.L. (1991) GABAA Receptor Subtypes: From Pharmacology to Molecular Biology, *FASEB J. 5,* 2916–2923.
19. Nielsen, M., Witt, M.R., and Thogersen, H. (1988) (^{3}H)Diazepam Specific Binding to Rat Cortex *In Vitro* Is Enhanced by Oleic, Arachidonic and Docosahexenoic Acid Isolated from Pig Brain, *Eur. J. Pharmacol. 146,* 349–353.
20. Levi de Stein, M., Medina, J.H., and De Robertis, E.E. (1989) *In Vivo* and *In Vitro* Modulation of Central Type Benzodiazepine Receptors by Phophatidylserine, *Mol. Brain Res. 5,* 9–15.
21. Witt, M.R., and Nielsen, M. (1994) Characterization of the Influence of Unsaturated Free Fatty Acids on Brain GABA/Benzodiazepine Receptor Binding *in Vitro, J. Neurochem. 62,* 1432–1439.

[Received January 10, 1995, and in final revised form July 17, 1995; Revision accepted October 17, 1995]

Effect of Marine Oils Supplementation on Coagulation and Cellular Activation in Whole Blood

Bjarne Østerud[a,*], Edel Elvevoll[b], Harald Barstad[b], Jan Brox[c], Hanne Halvorsen[a], Karin Lia[a], Jan Ole Olsen[a], Ragnar L. Olsen[b], Charlotte Sissener[a], Øystein Rekdal[d], and Eva Vognild[a]

Departments of [a]Biochemistry and [d]Biotechnology, Institute of Medical Biology, University of Tromsø, [b]Norwegian Institute of Fisheries and Aquaculture Ltd., 9037 Tromsø and [c]Hammerfest Hospital, 9600 Hammerfest, Norway

ABSTRACT: A study was performed to explore the effects of supplemental intake of various marine oils known to be part of the Eskimo diet. Healthy men and women (134) were randomly selected to consume 15 mL/d of oil from blubber of seal, cod liver, seal/cod liver, blubber of Minke whale, or no oil for ten weeks. Total cholesterol was unchanged in the oil groups, whereas high density lipoprotein cholesterol increased 7% in the seal/cod liver oil (CLO) group ($P < 0.05$) and 11% in the whale oil group ($P < 0.005$). Triacylglycerol was significantly reduced in the CLO group only. The concentration of prothrombin fragment 1 + 2 was reduced 25% ($P < 0.05$) after whale oil supplementation. No change in fibrinogen or factor VIIc was detected. Tumor necrosis factor generation in lipopolysaccharide (LPS)-stimulated blood was 30% reduced after whale oil ($P < 0.05$), but was unaffected by intake of seal or CLO. The LPS-induced tissue factor activity in monocytes was reduced to a significant degree only in the seal/CLO group (34%) and whale oil group (35%) ($P < 0.05$). The most dramatic change in thromboxane B_2 in LPS-stimulated blood was seen after whale oil intake with 44% reduction ($P < 0.01$). Supplementation of a regular diet with a combination of seal oil and CLO and especially with whale oil seems to have beneficial effects on several products thought to be associated with cardiovascular and thrombotic diseases.

Lipids 30, 1111–1118 (1995).

In 1978 Dyerberg *et al.* (1) suggested that the high content of n-3 polyunsaturated fatty acids present in the traditional Greenland Eskimo diet offers protection against atherosclerosis and thrombosis. This inspired a number of scientists throughout the world to carry out epidemiological studies on the importance of dietary intake of fish and fish oil and to explore the effects of n-3 polyunsaturated fatty acids (PUFA) on various important biological systems.

*To whom correspondence should be addressed.

Abbreviations: CLO, cod liver oil; CV, coefficient of variation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FVIIc, factor VII coagulant activity; F1 + 2, prothrombin fragment 1 + 2; HDL, high density lipoprotein; LPS, lipopolysaccharide; PAF, platelet activating factor; PDGF, platelet derived growth factor; PUFA, polyunsaturated fatty acids; TF, tissue factor; TG, triacylglycerol; TNF, tumor necrosis factor; TxA, thromboxane A; TxB, thromboxane B.

Although several recent studies have shown that a large intake of n-3 fatty acids is beneficial with regard to lowering blood pressure (2), reducing triacylglycerols (3), and modulation of cell function and cell reactivity to external stimuli (4–7), the final conclusion depends on more controlled studies (8). One of the reasons for this is that when epidemiological data relating fish consumption to cardiovascular disease were corrected for covariants, the results were controversial (9,10). The putative role of n-3 fatty acids from fish oil on the development of coronary heart disease has been questioned (11).

Although the low death rate from coronary heart disease among the Eskimos of Greenland has been suggested to stem in large part from their consumption of fish, one should keep in mind that the daily diet of Eskimos living in the traditional way consists of substantial quantities of meat and fat (blubber) from seals and whales (12). The fat composition of seals and especially whales differs significantly from that of fish. (13). Hence, one may ask whether oil from these animals may be better candidates than fish oil in explaining the apparent protective effect of the Greenland Eskimo diet. So far no controlled studies pertinent to any effects of these oils have been performed.

In this study we have raised the question as to whether seal and whale oils are more effective than cod liver oil (CLO) in changing biological parameters that might be important in explaining low incidence of coronary heart disease, asthma, and psoriasis among Greenland Eskimos (14). In order to address this question, we have measured some related parameters that are probably associated with, or mediators of, inflammatory reactions, atherogenesis, and thrombus formation before and after ten weeks of daily dietary supplemention with oils from fish, seal, and whale. In addition to determining serum lipids, fatty acid, total cholesterol, high density lipoprotein (HDL) cholesterol, and triacylglycerol (TG) levels, we measured the coagulation factors fibrinogen and factor VII, both of which have been proposed to be risk factors in ischemic heart disease (15,16).

It has been established that monocytes play a central role in atherosclerosis through their adhesion to a damaged endothelium, thus inducing and sustaining atherosclerosis. In

Copyright © 1995 by AOCS Press

the present study, the reactivity of monocytes in blood was examined by measuring lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production and LPS-induced tissue factor (TF) activity in monocytes of whole blood *in vitro*. This test system is used since we have seen that LPS stimulation of whole blood is a sensitive way to measure the ability of cells to be activated under physiological conditions. Thus the cellular responsiveness to LPS is quite constant over time but varies considerably between individuals (17,18). The response of blood cells to LPS *in vitro* correlates well with the activation during physical exercise (19).

Thromboxane A_2 (TxA_2), which induces platelet aggregation and vasoconstriction, is a major metabolite of activated platelets. Its pathophysiological importance is essentially still unknown, although inhibitors of the synthesis of TxA_2 decreased cholesterol-induced atherosclerotic foam lesions in rabbits (20). In the present study, thromboxane B (TxB_2), the stable metabolite of TxA_2, was measured in LPS-stimulated blood.

MATERIALS AND METHODS

One hundred and thirty-four healthy Norwegians living in Tromsø (72 men and 62 women), median age 29 years and range 18–60, gave informed consent to supplement their usual diet with daily consumption of marine oils known to be part of the Greenland Eskimo diet. They were randomly selected to consume 15 mL/d of Harp seal blubber oil (n = 27), CLO (n = 26), oil from blubber of Minke whale (n = 26), seal blubber oil/CLO (n = 27), and no oil (controls, n = 28) for ten weeks. The seal oil/CLO was a 1:1 volume mixture of these two oils. The blubber of whale was obtained from the animals hunted for research purposes in Norway in 1992 which was approved by the International Whaling Commission (Histon Cambridge, United Kingdom). The groups were all run in parallel. The various groups consisted of 16 men and 12 women (median age 26 and range 18–56 in control group); 16 men and 11 women (median age 29 and range 21–60) in seal oil group; 13 men and 13 women in CLO group (median age 28 and range 19–54); 13 men and 14 women in seal/CLO group (median age 34 and range 21–57); and 14 men and 12 women in whale oil group (median age 26 and range 21–54). There were seven smokers in the control group and seal oil group, eight smokers in the seal/CLO and whale oil groups, and six smokers in the seal oil group. The participants were told not to change their regular diet during the study. None of them had been using supplements of n-3 fatty acids for four months prior to the study. After the study was finished, they conveyed how much of the oil they had consumed. None of the subjects were taking any chronic or acute medication (including aspirin) one month before or during the study. Prior to each blood sampling, the participants were not allowed to drink alcohol or perform any strenuous physical activity for 48 h.

The fatty acid composition of the oils was determined by gas–liquid chromatography using on-column injection and flame-ionization detector detection. The participants were not told which oil they were drinking, and the code was opened after completing analysis of the samples. The procedures followed were in accord with the Helsinki Declaration of 1975 as revised in 1983.

Twenty-five milliliters of venous blood was withdrawn from fasting subjects between 8:00 a.m. and 10:00 a.m. on three different days before the start of supplementation and ten weeks after. The blood samples in the syringe were immediately portioned into the following tubes: a 5-mL polycarbonate tube containing 10 U heparin/mL blood, tubes containing either 3.2% sodium citrate or 2% Na_2EDTA in a 9:1 blood/anticoagulant ratio, and serum glass tubes.

Citrated blood was centrifuged immediately at $1200 \times g$, plasma pipetted off, and stored at −80°C. EDTA blood was analyzed within two hours in a Coulter Counter (Luton, England), STKR for blood cell counts. Serum tubes were incubated at room temperature for two hours, centrifuged at $1200 \times g$ for 10 min and the resultant sera stored at −80°C. The samples were assayed in a bulk, i.e., samples from one individual were assayed in one series.

Stimulation of whole blood in vitro. Heparinized blood was divided into 1-mL aliquots in polycarbonate tubes and LPS (LPS, 026:B6; Difco, Detroit, MI) added to a final concentration of 5 ng/mL blood followed by incubation at 37°C in an incubator shaker (200 rpm) for 2 h. The LPS stimulation was stopped by adding 0.1 mL 2% Na_2EDTA per mL blood. Part of the LPS-stimulated blood was immediately centrifuged at $1200 \times g$ for 10 min, and the resultant plasma pipetted off and frozen in aliquots at −80°C for later quantitation of TNF-α and TxB_2. Another part of the stimulated blood was subjected to isolation of mononuclear cells on Lymphoprep (Nycomed, Oslo, Norway) as described earlier (21). The mononuclear cells were resuspended in 0.15 M NaCl and frozen at −80°C until testing for LPS induced TF activity in monocytes.

Determination of TNF-α. TNF-α produced and secreted in LPS-stimulated blood was measured using a commercially available DuPont Human tumor necrosis factor-alpha ELISA kit (DuPont, NEN Research Products, Boston, MA). In the range we used this assay, its intra-assay coefficient of variation (CV) was 2.3%.

Quantitation of TF activity. TF activity was measured in frozen and thawed monocyte-samples in a two-stage clotting assay based on the ability of TF to accelerate the activation of factor X by factor VIIa as described (22). The activities measured in the samples were expressed in milliunits of crude extract of TF prepared from human brain. A 1:5000 dilution of this standard gave a clotting time of 25 s in the assay system. The samples were diluted 100- to 500-fold just before testing. Platelets and lymphocytes did not affect the clotting time of this assay.

TxB_2 determination. TxB_2, the stable product of TxA_2, was measured in plasma of LPS-stimulated blood using a thromboxane B_2 (^3H) radioimmunoassay kit (NEN Research Products). In this method, separation of the antibody–antigen complexes from free antigen is achieved by adsorption of the free tracer onto activated charcoal. After centrifugation, the

supernatant containing the antibody–antigen complexes is decanted into a counting vial, and scintillation cocktail is added. The cross reactivity in this kit is 100% for TxB_2, whereas cross reactivities to related compounds are: PGD_2, 0.3%; PGE_2, 0.04%; PGF_2, 0.03%; 6-keto-PGF_1, <0.5%; and PGA_2, <0.05%.

Coagulation factors. Prothrombin fragment 1 + 2 (F1 + 2) was measured in citrated plasma using the Enzygnost F1 + 2 ELISA kit from Behring (Behringwerke AG, Marburg, Germany) according to the manufacturer's instructions. The test system has a lower detection limit of 0.02 nmol/l F1 + 2. In the range 0.12 to 6.7 nmol/L, the CV in the series (intra-assay CV) was found to be 6 to 9%. The reproducibility of F1 + 2 measurements in plasma was 88 to 110% according to the manufacturer.

Factor VII coagulant activity (FVIIc) was measured in a one-stage clotting assay utilizing factor VII-deficient plasma and a human brain thromboplastin reagent (23). Fibrinogen was quantitated according to the method described by Inada *et al.* (24).

Lipids in serum. Serum samples were analyzed for TG, cholesterol, and HDL-cholesterol using commercially available enzyme-kits from Boehringer (Mannheim, Germany), in combination with an RA-1000 automatic analyzer (Technion RA-1000; Bayer Norway, Hagan, Norway).

Fatty acids in serum. The fatty acids were extracted from serum by the method of Folch and co-workers (25) and analyzed by gas–liquid chromatography using on-column injection and flame-ionization detector detection.

Statistical analysis. Data are reported as mean ± standard error of mean. All the statistical data presented for serum lipids, fibrinogen, FVIIc, prothrombin F1 + 2, TNF-α, TF, and TxB_2 was analysed by using a two-way repeated measures analysis of variance. Statistical analysis of differences between the control and the various groups in fatty acid composition of serum after ten weeks' supplementation with marine oils were performed with Dunnett's *t*-test. A *P*-value less than 0.05 was considered significant.

RESULTS

Fatty acid composition of the marine oils and of serum from the volunteers after ten weeks' supplementation with various marine oils. Part of the fatty acid composition of the marine oils is given in Table 1. This table shows that fatty acid composition of Harp seal oil and CLO is relatively similar, whereas Minke whale oil contains less PUFA and a little more monounsaturated fatty acids than the two other oils.

Compared with the control group, there were small differences in the saturated fatty acids of serum after ten weeks' intake of oils in the various groups (Table 2). Relatively small differences also were seen in the monounsaturated fatty acids although the level of 20:1 was significantly higher in all groups compared to the control group except the seal oil group, whereas the 24:1 was significantly higher in all four groups. Noteworthy is the lower level of 18:1 which was only significant in the CLO and CLO/seal oil groups. Among the PUFA, there was a significantly lower level of 18:2 in the seal oil and CLO groups as compared to the control group. As expected from the fatty acid profile in the various oils, the difference from the control group was highest in 20:5 and 22:6. Thus, 20:5 was 223, 262, 295, and 126% higher, respectively, in the seal oil, CLO, seal/CLO, and whale oil groups, whereas a similar, although smaller, difference of 22:6 was found (71, 83, 102, and 41% in the same order). There was a trend (not significant) of higher 20:5 and 22:6 in the combined seal/CLO group than in the groups with either seal oil or CLO alone.

TABLE 1
Types of Fatty Acids in the Marine Oils Used for Supplements (% w/w)

	Minke whale oil	Harp seal oil	Cod liver oil
Saturated			
14:0	5.3	5.1	3.6
16:0	7.9	8.2	9.5
18:0	1.8	0.9	2.0
20:0	0.3	0.2	0.1
Total	15.3	14.4	15.2
Monounsaturated			
16:1n-9	8.8	11.4	7.3
16:1n-7	0.2	0.3	0.3
18:1n-9	16.3	16.5	14.4
18:1n-7	1.8	4.0	4.5
20:1n-11	1.7	2.0	0.8
20:1n-9	15.3	9.0	10.6
22:1n-11	9.8	4.0	6.6
22:1n-9	0.9	0.6	0.2
Total	54.8	47.8	44.7
Polyunsaturated			
18:2n-6	1.4	1.7	1.6
18:3n-6	0.1	0.1	0.2
18:3n-3	1.2	1.2	1.3
18:4n-3	1.6	3.1	2.6
20:4n-6	0.3	0.3	0.4
20:4n-3	1.1	0.6	0.7
20:5n-3	4.3	6.8	9.5
21:5n-3	0.3	0.5	0.4
22:5n-6	0.2	0.3	0.2
22:5n-3	2.3	3.7	1.1
22:6n-3	7.9	11.1	13.1
Total	20.7	29.4	31.1
n-3 Polyunsaturated	18.7	27.0	28.7

Serum lipids. Total cholesterol levels were not significantly altered in any of the groups after ten weeks' supplemental intake of marine oils (Table 3), whereas HDL-cholesterol increased 7% in the seal/CLO group ($P < 0.05$) and 11% in the whale oil group ($P < 0.005$). TG were significantly reduced (23%) in the CLO group only ($P < 0.05$).

Fibrinogen and FVIIc. Large-scale epidemiological studies have indicated that the plasma content of fibrinogen and FVIIc are powerful risk factors for ischemic cardiovascular disease (15,16). In the present study we found no effect on FVIIc levels or fibrinogen in any of the oil groups (Table 4).

TABLE 2
The Fatty Acid Levels in Serum after Ten Weeks' Supplementation with Various Marine Oils (% w/w) (mean value ± SEM)[a]

Fatty acid	Control (n = 28)	Seal (n = 27)	CLO (n = 26)	CLO/seal (n = 27)	Whale (n = 26)
12:0	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.08 ± 0.02
14:0	0.90 ± 0.04	0.83 ± 0.05	0.79 ± 0.05	0.85 ± 0.06	0.86 ± 0.05
15:0	0.24 ± 0.02	0.25 ± 0.01	0.23 ± 0.02	0.26 ± 0.01	0.22 ± 0.02
16:0	18.87 ± 0.35	18.83 ± 0.28	17.96 ± 0.34	18.45 ± 0.33	17.97 ± 0.42
17:0	8.62 ± 0.28	9.13 ± 0.28	8.84 ± 0.25	8.62 ± 0.29	8.82 ± 0.28
18:0	5.61 ± 0.11	5.89 ± 0.14	5.86 ± 0.13	5.92 ± 0.11	5.59 ± 0.11
20:0	2.33 ± 0.19	2.16 ± 0.19	2.70 ± 0.22	2.11 ± 0.19	2.61 ± 0.18
22:0	0.43 ± 0.02	0.48 ± 0.03	0.47 ± 0.02	0.50 ± 0.02	0.46 ± 0.02
16:1	1.59 ± 0.08	1.58 ± 0.07	1.26 ± 0.04[b]	1.45 ± 0.10[b]	1.42 ± 0.09
18:1	17.52 ± 0.46	16.49 ± 0.36	15.64 ± 0.30[b]	15.52 ± 0.42[b]	16.17 ± 0.45
20:1	0.18 ± 0.04	0.23 ± 0.02	0.30 ± 0.03[b]	0.29 ± 0.03[b]	0.32 ± 0.09[b]
22:1	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
24:1	0.34 ± 0.02	0.44 ± 0.03[b]	0.46 ± 0.02[b]	0.50 ± 0.02[b]	0.48 ± 0.02[b]
18:2n-6	25.12 ± 0.48	23.11 ± 0.54[b]	22.94 ± 0.46[b]	23.47 ± 0.54	24.57 ± 0.54
18:3n-3	0.50 ± 0.03	0.43 ± 0.03	0.41 ± 0.01[b]	0.46 ± 0.02	0.44 ± 0.02
20:3n-3	1.09 ± 0.05	0.70 ± 0.04[b]	0.73 ± 0.04[b]	0.69 ± 0.03[b]	0.70 ± 0.04[b]
20:4n-6	3.63 ± 0.14	3.43 ± 0.11	3.38 ± 0.09	3.33 ± 0.11	3.48 ± 0.12
20:4n-3	0.90 ± 0.14	2.91 ± 0.23[b]	3.26 ± 0.22[b]	3.56 ± 0.26[b]	2.04 ± 0.16[b]
22:5n-3	0.32 ± 0.02	0.53 ± 0.03[b]	0.47 ± 0.02[b]	0.55 ± 0.03[b]	0.45 ± 0.02[b]
22:6n-3	1.81 ± 0.11	3.10 ± 0.13[b]	3.32 ± 0.13[b]	3.66 ± 0.16[b]	2.55 ± 0.09[b]

[a]Statistical differences (Dunnett's *t*-test) between the control group and the various marine oil groups are indicated; CLO, cod liver oil.
[b]$P < 0.05$.

F1 + 2 in plasma. F1 + 2 is an activation peptide released after proteolysis of prothrombin by factor Xa (26). The occurrence of F1 + 2 in plasma has been shown to serve as a useful indication of coagulation activation *in vivo*. The results depicted in Table 4 show that the level of F1 + 2 was significantly reduced in the whale oil group (from 0.99 ± 0.08 to 0.74 ± 0.04, $P < 0.05$). No significant changes were observed in any of the other groups.

Generation of TNF-α and TF activity in monocytes of LPS-stimulated blood. Stimulation of whole blood with a low concentration of LPS (5 ng/mL) has been used as a method to test the reactivity of cells and platelets in blood. Measurements of TNF-α in plasma obtained from blood incubated for 2 h with LPS, before and after ten weeks of the different diet supplements, revealed a significantly reduced level in the whale oil group (Fig. 1). Thus, the TNF-α level was about 30% lower after the oil intake was started than before (from 2.3 ± 0.3 to 1.6 ± 0.2 ng/mL, $P < 0.05$). Although there also was a 17% reduction in TNF-α in the seal/CLO group, this was not statistically significant since there also was a 12% reduction in the control group.

The LPS-induced TF activity in monocytes of whole blood was significantly reduced in all groups, including the control group (Fig. 2), but to a different degree. Thus, the highest reduction in LPS-induced TF activity was found in the monocytes of the subjects in the seal/CLO group and the whale oil group, i.e., 34.4 and 34.9%, respectively ($P < 0.05$). The reduction in the seal oil group (11.5%) and CLO group (12.6%) was similar to that of the control group (14.2%).

Generation of TxB_2 in LPS-stimulated blood. LPS-stimulated blood generates TxB_2 mainly from the platelets (27). This product, appearing in LPS-stimulated blood, was reduced in all four groups after ten weeks of oil supplementation (Fig. 3). The reduction in TxB_2 production in the seal oil,

TABLE 3
Serum Lipids Before and After Ten Weeks' Supplementation with Marine Oils (mean value ± SEM)[a]

	TG (mmol/L)		Tot-Chol (mmol/L)		HDL-Chol (mmol/L)	
	Before	After	Before	After	Before	After
Control (n = 28)	1.18 ± 0.12	1.20 ± 0.08	4.94 ± 0.23	4.75 ± 0.23	1.36 ± 0.09	1.36 ± 0.09
Seal (n = 27)	1.20 ± 0.10	1.06 ± 0.11	5.18 ± 0.22	5.04 ± 0.23	1.31 ± 0.04	1.37 ± 0.04
CLO (n = 26)	1.28 ± 0.16	0.98 ± 0.05[a]	5.29 ± 0.22	5.12 ± 0.22	1.25 ± 0.05	1.32 ± 0.07
Seal/CLO (n = 27)	1.29 ± 0.15	1.07 ± 0.11	5.30 ± 0.21	5.35 ± 0.21	1.36 ± 0.07	1.46 ± 0.08[a]
Whale (n = 26)	1.10 ± 0.08	1.02 ± 0.08	5.12 ± 0.21	5.18 ± 0.24	1.27 ± 0.06	1.41 ± 0.07[b]

[a]Compared to the changes in the control group, triacylglycerol (TG) was significantly reduced in the CLO group, $P < 0.05$, and HDL-Chol was significantly increased in the seal/CLO group[a] ($P < 0.05$) and whale oil group[b] ($P < 0.005$) (two-way analysis of variance). There were no significant differences between the various groups in base line values in any of the parameters. Tot-Chol = total cholesterol; HDL-Chol = high density lipoprotein-cholesterol. See Table 2 for other abbreviation.

TABLE 4
Coagulation Parameters Before and After Ten Weeks' Supplementation with Various Marine Oils (mean values ± SEM)[a]

Test parameter	Control (n = 28)		Seal oil (n = 27)		CLO (n = 26		CLO/seal oil (n = 27)		Whale oil (n = 26)	
	Before	After	Before	After	Before	After	Before	After	Before	After
FVIIc (U/mL)	1.08 ± 0.07	1.07 ± 0.07	1.23 ± 0.09	1.14 ± 0.08	1.16 ± 0.06	1.15 ± 0.10	1.21 ± 0.06	1.23 ± 0.07	1.20 ± 0.07	1.18 ± 0.06
Fibrinogen (g/L)	2.6 ± 0.1	2.4 ± 0.1	2.8 ± 0.2	2.6 ± 0.2	2.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.3 ± 0.1
F1 + 2 (nmol/L)	0.85 ± 0.06	0.80 ± 0.05	0.85 ± 0.05	0.82 ± 0.05	0.85 ± 0.06	0.78 ± 0.06	0.85 ± 0.06	0.75 ± 0.04	0.99 ± 0.08	0.74 ± 0.04[b]

[a]FVIIc = Factor VII coagulant activity; F1 + 2 = prothrombin fragment 1 + 2; see Table 2 for other abbreviation.
[b]$P < 0.05$ (compared to the change in the control group).

CLO, seal oil/CLO, and whale oil groups was 35.8, 33.8, 28.0, and 43.7%, respectively. In the whale oil group, the TxB_2 was reduced from 16.7 ± 2.0 to 9.4 ± 1.6 ng/mL ($P < 0.01$).

DISCUSSION

The results of the present study show that supplementation of an otherwise-unrestricted diet with moderate amounts of certain oils from marine fat, known to be part of the Greenland Eskimo diet, is effective in reducing the reactivity of blood cells and reducing the activation of coagulation in a favorable nonthrombotic direction. Surprisingly, intake of seal oil or CLO alone had only a relatively small effect. In contrast, the whale blubber oil alone appears to be superior in this regard. Interestingly, seal oil and CLO in combination seemed to be more effective than either of the two oils taken separately.

Weak LPS stimulation of whole blood has been used in our laboratory for more than ten years as a model for the reactivity of blood cells (18). This whole blood system may be less sensitive to changes in diet than seen in regular cell cultures, as we failed to find any significant change in LPS-induced TF and TNF-α after eight weeks' supplementation with 4 g/d of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) (Østerud, B., unpublished data). However, we believe that this *ex vivo* system is more relevant to *in vivo* conditions than artificial cell culture systems in studying changes in blood cells as a consequence of marine oil supplementation in the diet.

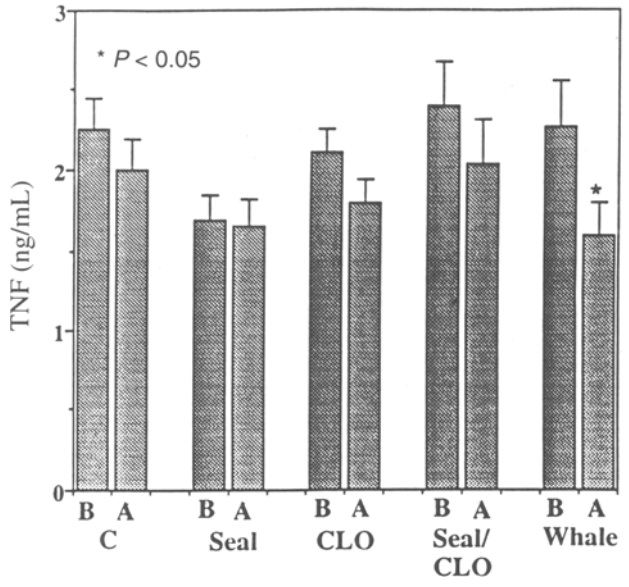


FIG. 1. Generation of tumor necrosis factor-α (TNF-α) by monocytes in lipopolysaccharide-stimulated whole blood before and after ten weeks' daily supplement with various marine oils. Values are means ± SEM. Compared to the change in the control group (C), only the change in TNF-α of the whale oil group was statistically significant ($P < 0.05$) (two-way analysis of variance). The TNF-α in the seal oil group was significantly lower in baseline than in all other groups. CLO = cod liver oil, B = before, A = after marine oil supplementation (n = 28 in control group, 27 in seal oil group, 26 in CLO group, 27 in seal/CLO group, and 26 in whale oil group).

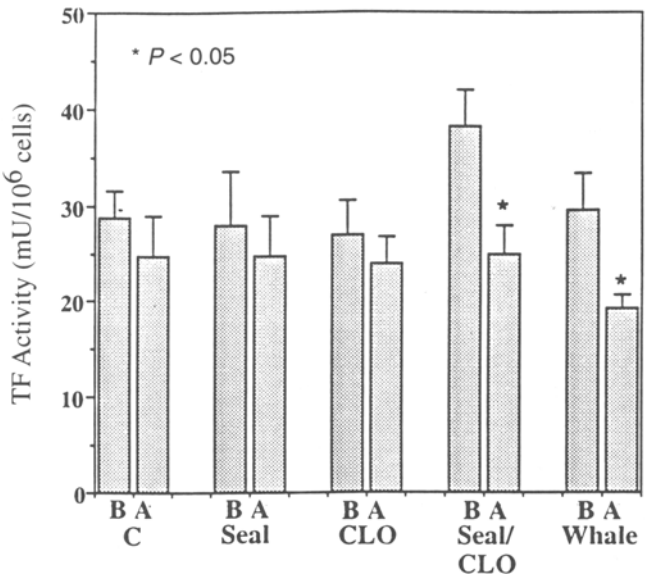


FIG. 2. Formation of tissue factor (TF) activity in monocytes in lipopolysaccharide-stimulated whole blood before and after ten weeks' daily supplement with various marine oils. Values are means ± SEM. Compared to the change of TF activity in the control group (C), the TF activity was significantly reduced in the seal/cod liver oil (CLO) group and the whale oil group (two-way analysis of variance). There was no significant difference in baseline values of the different groups (n = 28 in control group, 27 in seal oil group, 26 in CLO group, 27 in seal/CLO group, and 26 in whale oil group).

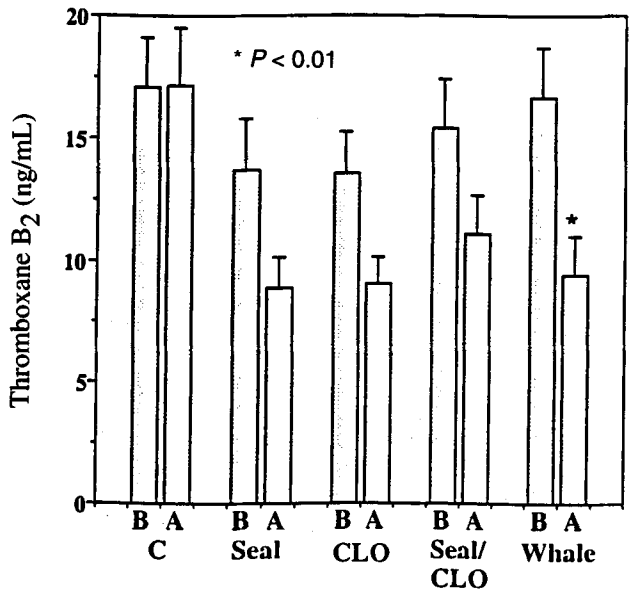


FIG. 3. Production of thromboxane B_2 (TxB_2) in lipopolysaccharide-stimulated whole blood before and after ten weeks' daily supplement with various marine oils. Values are means ± SEM. Compared to the control group (C), TxB_2 was significantly reduced only in the whale oil group (two-way analysis of variance). There was no significant difference in base line values between the different groups [n = 28 in control group, 27 in seal oil group, 26 on cod liver oil (CLO) group, and 27 in whale oil group].

The present study shows that supplementation of a regular diet with moderate amounts of whale oil reduced LPS-induced TNF-α and TF activity production in monocytes 30–40% on average, and TxB_2 production by about 44%. The smaller effect obtained by intake of seal oil or CLO alone (no significant change in TNF-α and TF activity vs. 30–35% reduction in TxB_2) may suggest that these oils lack factor(s) present in whale oil. Although the baseline values of TF are much higher in the seal/CLO group than in the other groups, the seal oil/CLO effect was also high in the subjects with low baseline TF activities. The large difference in baseline values of TF activity is caused by the great variation between individuals in responding to a low dose of LPS. Thus, a high responder will always have high values, whereas a low responder will have low values (18). TNF-α is believed to play a critical role as an early response cytokine in cellular activation and the propagation of the inflammatory response [for review, see Strieter *et al.* (28)]. Atherosclerosis is today suggested to reflect a defense system gone awry (29), and TNF-α may thereby be associated with the atherosclerotic process. Furthermore, tissue factor is the most potent trigger of blood coagulation, and its localization in macrophages in atherosclerotic plaques probably accounts for the instant thrombus formation following plaque rupture. Thus, downregulation of both TNF-α and TF production in monocytes/macrophages may be quite beneficial in the prevention of thrombosis and atherosclerosis. In this context, it is of interest finding that women produce 39% less TNF-α than men using the same whole blood test system (30) and that estrogen replacement for a year in postmenopausal women caused a 35% reduction in LPS-induced TNF-α (31).

A reduction in cellular activation products has been documented after dietary supplementation with relatively large amounts of PUFA, of which the n-3 fatty acids, EPA and DHA, have been suggested to be of crucial significance (4,5). The antiatherogenic effects of n-3 fatty acids have been suggested to be multifunctional (32). As seen in Table 1, the most striking difference between the fatty acid compositions of seal oil, CLO, and whale oil is that the content of n-3 fatty acids in the whale oil is only approximately 60–65% of that of seal oil and CLO. The amount of EPA (20:5n-3) in the whale oil is less than half of the amount found in CLO. In addition, there is approximately 55% monounsaturated fatty acids in the whale oil, which is 10% more than that of CLO and seal oil. It is therefore questionable whether the n-3 fatty acids alone in the whale oil can be responsible for the apparent beneficial effect of this oil.

The effect of different marine oils on the TG level in serum is quite noteworthy, since CLO appeared to be the only oil that caused a significant reduction. The reduction of TG by the CLO corresponds relatively well with the content of n-3 fatty acids in this oil and dose responses reported in the literature (33). The question arises as to why seal oil does not produce such an effect, since its fatty acid composition is very similar to that of CLO (Table 1). Since reduction in TG also has been documented using relatively pure EPA as a dietary supplement, it has been assumed that this fatty acid is the main component in CLO causing the reduction in TG (34). Based on the content of EPA in CLO, seal oil, and whale oil, one should have expected the reduction of TG in the seal oil group to be about 17% (instead of 12%) and about 11% (instead of 7%) in the whale oil group. This discrepancy in response is probably attributed to the localization of EPA in the TG molecule. Whereas EPA is mainly found in the 1- and 3-positions of seal oil TG, it is equally distributed in positions 2 and 3 in CLO (35). Thus EPA is about five times more likely to be found in position 2 of the TG in CLO as compared to those of seal oil.

The n-3 fatty acids may have low or no effect as cholesterol-lowering modulators (33). None of the oils used in our study appeared to have any effect on the total cholesterol concentration in serum. However, whale oil and the seal/CLO combination caused a small but significant increase in HDL-cholesterol. This observation may be relevant to the finding by Dyerberg *et al.* (36) that levels of low density lipoprotein-cholesterol were significantly lower and that levels of HDL-cholesterol were higher among Eskimos than among Danes.

Our data may indicate a reduction in the ongoing *in vivo* activation of blood coagulation, as judged by F1 + 2 levels in plasma, after marine oil supplementation. Interestingly, such an effect was not observed using either CLO, seal oil, or seal oil/CLO, but it was significant following the intake of whale oil (25% reduction). Since the F1 + 2 level reflects proteolysis of prothrombin by factor Xa, one may speculate that less factor Xa is generated after whale oil supplementation due to

reduced TF available for the interaction with factor VII. It also could reflect impaired prothrombinase complex formation due to reduced activation of the platelets *in vivo*.

FVII and fibrinogen have been claimed to be powerful risk factors for ischaemic cardiovascular diseases. In our study we did not observe any significant change in FVIIc and fibrinogen in plasma after any of the oil supplements. In the great majority of trials, plasma fibrinogen levels are not significantly affected by dietary fish oil, but in a couple of studies intake of fish oil concentrates resulted in a significant reduction of the plasma fibrinogen level (for review, see Ref. 37).

In conclusion, daily dietary supplementation with whale oil alone or seal/CLO in combination appeared to have the most pronounced effects on some selected biological parameters thought to play a role in cardiovascular disease. Although we did not perform any analysis of diet or dietary effect of supplementation, we are quite confident that the changes observed in our test model are caused by the oil supplementation. Pilot studies and an extensive experience with single individuals on various marine oils have all given results in accordance with the present study.

The difference in the effects of whale and seal oils argues against the position in TG as being of relevance to the superiority of whale oil, since the n-3 fatty acids are mainly in the 1- and 3-position of both these oils. However, the difference in effects of CLO and seal oil as compared to the beneficial effects by these oils in combination may indicate two things: (i) there are two (or more) essential factors needed and that whale oil contains both, but seal and fish oils contain one of each, or (ii) the beneficial component(s) of whale oil is unrelated to the beneficial components of CLO/seal oil in combination. The effect of whale oil is probably not mediated by n-3 fatty acids alone (or at all) as the content of these fatty acids is relatively low in whale oil and corresponds to an intake of only 1.8 g n-3 fatty acids per day. Our study may strongly indicate that in addition to n-3 fatty acids, there may be another component present in the Greenland Eskimo diet that might explain its apparent protective effect against atherosclerosis and thrombosis. As this is the first controlled study to survey a number of parameters potentially influenced by components of the Eskimo diet, we realize that more studies are required to clarify the role of oils from marine blubbers as a beneficial supplement to the diet in the prevention of coronary heart disease and thrombosis. Such studies are already in progress in our laboratory.

ACKNOWLEDGMENTS

The technical assistance of Eli Berg and Elisabet Rasch is gratefully acknowledged. The authors are indebted to Jens Petter Wold for carrying out the statistical work. This study was supported by the Norwegian Research Council, the Norwegian Council on Cardiovascular Diseases, and the Nansen Foundation.

REFERENCES

1. Dyerberg, J., Bang, H.O., Steffensen, E., Moncada, S., and Vane J.R. (1978) Eicosapentaenoic Acid and Prevention of Thrombosis and Atherosclerosis, *Lancet 2*, 117–119.
2. Bønaa, K.H., Bjerve, K.S., Straume, B., Gram, T.T., and Thelle, D. (1990) Effect of Eicosapentaenoic and Docosahexaenoic Acids on Blood Pressure in Hypertension. A Population-Based Intervention Trial from the Tromsø Study, *N. Eng. J .Med. 322*, 795–801.
3. Harris, W.S., Zucker, M.L., and Dujovne, C.A. (1988) Omega-3 Fatty Acids in Hypertriglyceridemic Patients: Triglycerides vs. Methyl Esters, *Am. J. Clin. Nutr. 48*, 992–997.
4. Lee, T.H., Hoover, R.L., and Williams, J.D., Sperling, R.I., Ravalese, J. III, Spur, B.W., Robinson, D.R., Corey, E.J., Lewis, R.A., and Austen, K.F. (1985) Effect of Dietary Enrichment with Eicosapentaenoic and Docosahexaenoic Acids on *in vitro* Neutrophil and Monocyte Leukotriene Generation and Neutrophil Function, *N. Eng. J. Med. 312*, 1217–1224.
5. Endres, S., Ghorbani, R., Kely, V.E., Georgilis, K., Lonneman, G., van der Meer, J.W., Cannon, J.G., Rogers, T.S., Klempener, M.S., Weber, P.C., Schaefer, E.J., Wolff, S.M., and Dinarello, C.A. (1989) The Effect of Dietary Supplementation with n-3 Polyunsaturated Fatty Acids on the Synthesis of Interleukin-1 and Tumor Necrosis Factor by Mononuclear Cells, *N. Eng. J. Med. 320*, 265–271.
6. Hansen, J.B., Olsen, J.O., Wilsgård, L., and Østerud, B. (1989) Effects of Dietary Supplementation with Cod Liver Oil on Monocyte Thromboplastin Synthesis, Coagulation and Fibrinolysis, *J. Int .Med. 225 Suppl. 1*, 133–139.
7. Fox, P.L., and DiCorleto, P.E. (1988) Fish Oils Inhibit Endothelial Cell Production of Platelet-Derived Growth Factor-Like Proteins, *Science 241*, 453–466.
8. Goodnight, S.H. (1993) The Effects of n-3 Fatty Acids on Atherosclerosis and the Vasculature Response to Injury, *Arch. Pathol. Lab. Med. 117*, 102–106.
9. Kromhout, D. (1989) n-3 Fatty Acids and Coronary Heart Disease: Epidemiology from Eskimos to Western Populations, *J. Int. Med. 225 Suppl. 1*, 47–51.
10. Combie, T.A., and Granditis, G. (1991) Dietary Polyunsaturated Fatty Acids and Mortality in the Multiple Risk Factor Intervention Trial (MRFIT), in *World Rev. Nutr. Diet* (Simopoulos, A.P., Kifer, R.R., and Barlow, S.M., eds.) Vol. 66, pp. 205–215, Karger, Amsterdam.
11. Kromhout, D., Bosschieter, E.B., and Coulander, C. deL. (1985) The Inverse Relation Beween Fish Consumption and Mortality from Coronary Heart Disease, *N. Eng. J. Med. 312*, 1205–1209.
12. Bang, H.O., Dyerberg, J., and Hjørne, N. (1976) The Composition of Food Consumed by Greenland Eskimos, *Acta Med. Scand. 200*, 69–73.
13. Padley, F.B., Gunstone, F.D., and Harwood, J.L. (1986) Marine Fish Oils and Whale Oils, in *The Lipid Handbook* (Gunstone, F.D., Harwood, J.L., and Padley, F.B., eds.) pp. 130–141, Chapman and Hall, London.
14. Kromann, N., and Green, A. (1980) Epidemioloogical Studies in the Upernavik District, Greenland, *Acta Med. Scand. 208*, 401–406.
15. Wilhelmsen, L., Svardsudd, K., Korsan-Bengtson, K., Larsson, B., Walin, L., and Tibblin, G. (1984) Fibrinogen as a Risk Factor for Stroke and Myocardial Infarction, *N. Eng. J. Med. 311*, 501–505.
16. Meade, T.W., Brozovic, M., Chakrabarti, R.R., Hannes, A.P., Imeson, J.D., Mellows, S., Miller, G.J., North, W.R.S., Stirling, Y., and Thompson, S.G. (1986) Haemostatic Function and Ischaemic Heart Disease: Principal Results of the Nortwick Park Heart Study, *Lancet 2*, 533–537.
17. Østerud, B., Olsen, J.O., and Wilsgård, L. (1990) The Role of Arachidonic Acid Release and Lipoxygenase Pathway in Lipopolysaccharide-Induced Thromboplastin Activity in Monocytes, *Blood Coagulation and Fibrinolysis 1*, 41–46.
18. Østerud, B. (1995) The High Responder Phenomenon: Enhance-

ment of LPS-Induced Tissue Factor Activity in Monocytes by Platelets and Granulocytes, *Platelets 6*, 119–125.
19. Østerud, B., Olsen, J.O., and Wilsgård, L. (1989) Effect of Strenuous Exercise on Blood Monocytes and Their Relation to Coagulation, *Med. Sci. Sports Exerc. 21*, 374–378.
20. Shrinska, V.A., Koieczowski, M., Gerrity, R.G., Galang, C.F., and Rebec, M.V. (1988) Suppression of Foam Cell Lesions in Hypercholesterolemic Rabbits by Inhibition of Thromboxane A_2 Synthesis, *Arteriosclerosis 8*, 359–367.
21. Østerud, B., and Bjørklid, E. (1982) Factor VII Associated with Monocytes in Endotoxin Stimulated Blood, *Biochem. Biophys. Res. Commun. 108*, 620–626.
22. Østerud, B. (1992) Platelet Activating Factor Enhancement of Lipopolysaccharide-Induced Tissue Factor Activity in Monocytes: Requirement of Platelets and Granulocytes, *J. Leuk. Biol. 51*, 462–465.
23. Østerud, B., and Rapaport, S.I. (1970) Synthesis of Intrinsic Factor X Activator. Inhibition of the Function of Formed Activator by Antibodies to Factor VIII and Factor IX, *Biochemistry 9*, 1854–1861.
24. Inada, Y., Okamoto, H., Kanai, S., and Sarajas, S. (1978) Faster Determination of Clottable Fibrinogen in Human Plasma: An Improved Method and Kinetic Study, *Clin. Chem. 24*, 351–353.
25. Folch, I., Lees, M., and Stanley, S.A. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
26. Teitel, J.M., Bauer, K.A., Lau, H.K., and Rosenberg, R.D. (1982) Studies of Prothrombin Activation Pathways Utilizing Radioimmunoassay for the F_2/F_{1+2} Fragment and the Thrombin-antithrombin Complex, *Blood 59*, 1086–1097.
27. Halvorsen, H., Olsen, J.O., and Østerud, B. (1993) Granulocytes Enhance LPS Induced Tissue Factor Activity in Monocytes *via* an Interaction with Platelets, *J. Leuk. Biol. 54*, 275–282.
28. Strieter, R.M., Kunkel, S.L., and Bone, R.C. (1993) Role of Tumor Necrosis Factor-Alpha in Disease States and Inflammation, *Crit. Care. Med. 21*, S415–S422.
29. Ross, R. (1993) Atherosclerosis: A Defense System Gone Awry, *Am. J. Pathol. 143*, 967–1002.
30. Balteskard, L., Brox, J.H., and Østerud, B. (1993) Thromboxane Production in the Blood of Women Increases After Menopause Whereas Tumor Necrosis Factor is Reduced in Women Compared to Men, *Atherosclerosis 102*, 91–98.
31. Aune, B., Øian, P., Omsjø, I., and Østerud, B. (1995) Hormone Replacement Therapy Reduces the Reactivity of Monocytes and Platelets in Whole Blood: Beneficial Effect on Atherogenesis and Thrombus Formation, *Am. J. Obst. Gynecol.*, in press.
32. Weksler, B.B. (1992) Platelets, Leukocytes and Atherosclerosis. Possible Links Between n-3 Fatty Acids and Protection from Atherosclerosis, in *Fish Oil and Vascular Disease* (De Caterina, R., Kristensen, S.D., and Schmidt, E.B., eds.) pp. 51–58, Springer-Verlag, Verona, Italy.
33. Harris, W.S. (1989) Fish Oils and Plasma Lipid and Lipoprotein Metabolism in Humans: A Critical Review, *J. Lipid Res. 30*, 785–807.
34. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M., and Berge, R.K. (1993) Docosahexaenoic Acid Shows No Triglyceride-Lowering Effects But Increases the Peroxisomal Fatty Acid Oxidation in Liver of Rats, *J. Lip. Res. 34*, 13–22.
35. Ackman, R.G., and Ratnayake, W.M.N. (1989) Fish Oils, Seal Oil, Esters and Acids—Are All Forms of Omega-3 Intake Equal?, in *Health Effects of Fish and Fish Oils* (Chandra, R.K., ed.) pp. 373–393, ARTS Biomedical Publishers & Distriburors, St. John's Newfoundland.
36. Dyerberg, J., Bang, H.O., and Hjørne, N. (1976) Fatty Acid Composition of the Plasma Lipids in the Greenland Eskimos, *Am. J. Clin. Nutr. 28*, 958–966.
37. Hornstra, G. (1992) The Effect of n-3 Fatty Acids on Blood Coagulation, in *Fish Oil and Vascular Disease* (De Caterina, R., Kristensen, S.D., and Schmidt, E.B., eds.) pp. 65–72, Springer-Verlag, Verona, Italy.

[Received February 1, 1995, and in final revised form September 28, 1995; Revision accepted October 12, 1995]

Molecular Architecture and Biophysical Properties of Phospholipids During Thermal Adaptation in Fish: An Experimental and Model Study

Elfrieda Fodor[a], Richard H. Jones[b], Csaba Buda[a], Klara Kitajka[c], Indranil Dey[c,d], and Tibor Farkas[a,*]

[a]Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, H-6701 Szeged, Hungary, [b]Department of Preventive Medicine & Biometrics, School of Medicine, University of Colorado Health Sciences Centre, Denver, Colorado 80262, [c]Bay Zoltán Institute for Applied Research, Szeged, Hungary, [d]Department of Pathology & Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

ABSTRACT: Phospholipids from livers of carps (*Cyprinus carpio* L.) adapted to winter (5°C) and summer (25°C) temperatures were isolated, and the fatty acid composition of total phospholipids, as well as molecular species composition of diacyl phosphatidylcholines and ethanolamines, were determined. Order parameter of 5-doxyl stearic acid and steady-state fluorescence anisotropy of different anthroyloxy fatty acids—[2-,12(*N*-9-anthroyloxy)stearic acid and 16(*N*-9-anthroyloxy)palmitic acid—embedded in native and synthetic (16:0/16:0, 16:0/22:6, 18:0/22:6, 18:1/22:6, 20:4/20:4, 22:6/22:6 phosphatidylcholines and 16:0/18:1, 18:1/22:6 phosphatidylethanolamines) phospholipid vesicles was also determined between −30 and 30°C and 5 and 30°C, respectively. There is an accumulation of 1-monoenoic, 2-polyenoic diacyl phosphatidylcholine and ethanolamine with a concomitant reduction of 1-stearoyl,2-docosahexaenoyl species in the cold-adapted state. Despite a 30% accumulation of long-chain polyunsaturated fatty acids in phospholipids in cold, there is only a 5°C downshift in the solid-gel to liquid-crystalline phase transition temperature (−8 vs. −13°C). Vesicles from total phospholipids of cold-adapted fish proved to be more disordered in all segments than from the warm-adapted ones when assayed using 2,12-(*N*-9-anthroyloxy)stearic and 16-(*N*-9-anthroyloxy)palmitic acid. Vesicles made from purified phosphatidylcholines showed the same pattern, but they were more disordered than the corresponding total phospholipids. This could be modelled using mixed phospholipid vesicles made of synthetic 16:0/22:6 phosphatidylcholine (75%) and either 18:1/22:6 phosphatidylethanolamine (25%) vs. 16:0/18:1 phosphatidylethanolamine (25%) and comparison of the anisotropy parameters of 100% 16:0/22:6 and 100% 18:1/22:6 phosphatidylcholine vesicles. Mixing either 16:0/18:1 (25%) or 18:1/22:6 (25%) phosphatidylethanolamines to 18:0/22:6 (75%) phosphatidylcholine shifted down or up, respectively, the transition temperature of vesicles compared to 100% 18:0/22:6 vesicles assayed by electron spin resonance spectroscopy using 5-doxylstearic acid. It is concluded that it is not the gross amount of long-chain polyunsaturated fatty acids in phospholipids, but rather their specific combination with *cis* Δ9 monounsaturated fatty acids in the position *sn*-1, especially in phosphatidylethanolamines, that is important in determining the physical properties of biomembranes in relation to adaptational temperature.

Lipids 30, 1119–1126 (1995).

Temperature is one of the most important environmental factors affecting the physiological activity of poikilotherms, such as fish. The first targets of temperature changes are the biomembranes which exist in a so-called liquid-crystalline state at body temperature (1). Thus the temperature can be regarded as a membrane perturbant to which the cell must respond to establish a new equilibrium between the environment and the physicochemical properties of their membraneous structures in order to survive under the new conditions, a process called homeoviscous adaptation (2). Membranes are assemblies of lipids and proteins (1), and the lipid components are considered to be responsible for maintaining their proper physicochemical properties. The concept of involvement of lipids in thermoregulation processes is based on the fact that the melting point of unsaturated fatty acids decreases with increases in the number of the double bonds in the molecule. Accumulation of long-chain polyunsaturated fatty acids in fish when the environmental temperature decreases has often been reported (3–5), and it has also been observed that the ratio of unsaturated to saturated fatty acids is inversely related to the temperature to which the animals are adapted. However, neither the thermotropic phase transition (6) nor the surface area (7) is a linear function of the number of double bonds present in the phospholipid molecules. In both cases, the introduction of the first double-bond results in most spectacular change in these parameters. Moreover, accumulation of long-chain polyenoic fatty acids is not necessarily linked to reduced temperatures, as demonstrated recently for marine and freshwater fish or shrimp evolutionarily

*To whom correspondence should be addressed.

Abbreviations: 16-AP, 16-(*N*-9-anthroyloxy)palmitic acid; 2-AS, 2-(*N*-9-anthroyloxy)stearic acid; 12-AS, 12-(*N*-9-anthroyloxy)stearic acid; ESR, electron spin resonance; PC, phosphatidylcholine(s); PE, phosphatidylethanolamine(s); R_{ss}, anisotrophy fluorescence; SA, summer-adapted; 5-SASL, 5-doxylstearic acid; WA, winter-adapted.

Copyright © 1995 by AOCS Press

adapted to the two temperature extremes (8,9). Furthermore, comparison of the order parameter of 5-doxylstearic acid (5-SASL) revealed only negligible differences between liver phospholipids of fish inhabiting either cold or warm seas for millions of years (6), suggesting that gross lipid unsaturation plays only a subordinate role in determining the overall fluidity of these structures, whereas characteristic differences were revealed in the structural organization of the bilayer (8–10). In this article, the composition and physical properties of phospholipids obtained from the livers of a freshwater fish (*Cyprinus carpio*), seasonally adapted to the two temperature extremes, is investigated. Hopefully the information obtained will contribute to a better understanding of the role that lipid components play in controlling membrane physical state and architecture in thermal adaptational processes.

MATERIALS AND METHODS

Animals. Fish (*Cyprinus carpio* L.), weighing 0.7–1.0 kg, were obtained from a local fish farm. The summer-adapted (SA) fish were collected at the end of the summer of 1993, when the water temperature was above 23°C for more than two months; and the winter-adapted (WA) fish were collected at the end of the winter of 1994 shortly after thawing. Livers of ten freshly killed animals were pooled for the analyses.

Extraction and analyses of lipids. Lipids were extracted according to Folch *et al.* (11). Phospholipids were separated by column chromatography on silicic acid, using chloroform to elute the neutral lipids and methanol to elute the polar lipids. Polar lipids were further separated into different subclasses by thin-layer chromatography on precoated silica gel plates (E. Merck, Darmstadt, Germany) according to Fine and Sprecher (12). Phosphatidylcholine(s) (PC) and phosphatidylethanolamine(s) (PE), after visualization by spraying with 0.5% 8-anilino-1-naphthalene sulfonic acid in 50% methanol, were eluted with chloroform/methanol (2:1, vol/vol) and stored in benzene containing 0.01% butylated hydroxytoluene at –20°C until further processing.

Fatty acid composition of total and individual phospholipids were determined using a Hitachi (Tokyo, Japan) Model 260-80 gas chromatograph equipped with dual flame-ionization detector after transmethylation in absolute methanol containing 5% HCl at 80°C for 2.5 h. Fatty acids were segregated on 10% free fatty acid phrase (Supelco, Bellefonte, PA) coated onto 100–120 mesh Supelcoport (Supelco). Quantitation was made using a data processor (Hitachi 260-80) connected to the gas chromatograph.

Molecular species determination of purified PC and PE were determined according to Takamura *et al.* (13). The diradylglycerols obtained by digestion with phospholipase C (*Bacillus cereus*) were converted to benzoyl derivatives, and the dinitroyl derivatives of diacylglycerols were separated into individual molecular species by high-performance liquid chromatography using a Hypersyl C_{18} column (250 × 4 mm i.d.). Acetonitrile and 2-propanol in a ratio of 80:20 served as mobile phase. Detection took place at 254 nm, and identification was made using authentic standards and on the basis of relative elution times given by Bell and Dick (14).

Spectroscopic techniques. Fluorescence anisotropy measurements were made on 250 μg phospholipids mixed with 10 μl of 1 μmol 2-(*N*-9-anthroxyloxy)stearic acid (2-AS), 12-(*N*-9-anthroloxy)stearic acid (12-AS), and 16-(*N*-9-antholoxy)palmitic acid (16-AP) (Molecular Probes Inc., Eugene, OR). This mixture of probes was evaporated onto the surface of a suitable test tube and suspended in Tris–HCl buffer, pH 7.4, according to Sen *et al.* (15). Measurement and calculation of steady-state fluorescence anisotropy (R_{ss}) were made according to Dey *et al.* (8). The SE of the determination was always less than 0.005.

Electron spin resonance (ESR) spectroscopy. Measurements were carried out by incorporating <1 mol% of 5-SASL (Sigma, St. Louis, MO) spin label into the phospholipid vesicles. The sample containing 1 mg of phospholipid was poured into sealed glass capillary (1 mm i.d.) and adjusted within a standard ESR tube under thermal regulation. Spectra were recorded from –30°C to 30°C using an ESC-106 ESR spectrometer (Brucker, Karlsruhe, Germany) under medium microwave power (10 mW) and low magnetic field modulation (1 mT). The outer hyperfine splitting ($2A_{max}$) of the spectra was used to characterize the membrane ordering state at a given temperature (16).

Determination of change-point temperatures. The procedure of Jones and Dey (17) was used to determine the thermotropic phase transition temperature of phospholipid vesicles. First, a straight line was fit to the data by least squares. Next, a model with a single change-point at an unknown location was fit by searching over the range of the data for the position of a change-point that minimized the sum of squares of residuals. A change-point is a point where the line suddenly changes slope, but the line is continuous. The model with a single break-point is tested statistically to determine if the break-point produces significant improvement in the fit to the data. The procedure is repeated by adding break-points until no significant improvement is obtained.

Chemicals. 1-Stearoyl, 2-docosahexaenoyl-, 1-palmitoyl, 2-docosahexaenoyl, 1-oleyl, 2-palmitoleyl, 1-oleoyl, 2-docosahexaenoyl PC, 1-palmitoyl, 2-oleyl-, 1-oleyl, and 2-docosahexaenoyl PE were from Avanti Polar Lipids, Inc. (Birmingham, AL). 2-AS, 12-AS, and 16-AP were from Molecular Probes, Inc. Acetonitrile and 2-propanol (high-performance liquid chromatography-grade) were products of Carlo Erba (Milan, Italy). All other reagents were of analytical grade.

RESULTS

WA carps accumulated 25% more long-chain polyunsaturated fatty acids (20:4n-6, 22:6n-3) in their total phospholipids at the cost of saturated palmitic and stearic acids, resulting in a drastic decrease in the ratio of saturated-to-unsaturated fatty acid (Table 1). Of the polyunsaturated fatty acids, 20:4n-6 gave the most sensitive response, showing an almost 100% accumulation. However, 22:6n-6 was less responsive. Accu-

TABLE 1
Fatty Acid Composition of Liver Total Phospholipids, Phosphatidylcholines, and Phosphatidylethanolamines of Carp Adapted to Summer (5°C) and Winter (25°C) Temperatures[a]

	Cold-adapted (wt%)			Warm-adapted (wt%)		
Fatty acid	TPL	CPG	EPG	TPL	CPG	EPG
14:0	0.8	7.0	3.4	0.1	10.6	11.6
16:0	14.4	23.0	15.0	25.5	32.1	21.9
16:1	6.6	11.5	1.1	3.9	5.5	0.4
18:0	8.8	5.1	9.1	17.7	9.2	15.0
18:1	16.0	15.5	15.1	14.2	17.1	10.2
18:2n-6	2.3	2.4	1.1	3.5	3.9	1.6
18:3n-3	2.7	1.5	4.3	1.7	0.3	0.6
20:4n-6	21.6	11.4	24.2	12.0	5.4	13.8
20:5n-3	1.1	0.4	1.2	1.2	1.6	1.0
22:4n-6	2.2	0.6	2.1	1.2	0.8	3.1
22:4n-3	3.4	1.5	3.0	2.7	0.3	3.5
22:5n-3	2.7	2.6	2.8	1.7	1.2	2.5
22:6n-3	17.4	16.9	17.4	14.5	12.0	14.7
S/US[b]	0.31	0.54	0.37	0.76	1.07	0.94

[a]Fatty acid methyl esters were analyzed by gas chromatography as described in the Materials and Methods section. TPL, total phospholipids; CPG, choline phosphoglycerides; EPG, ethanolamine phosphoglycerides.
[b]Ratio of saturated-to-unsaturated fatty acids.

mulation of 20:4n-6, which was not observed in a previous study (18), might be explained by possible abundance of linoleic acid in their natural food in this particular case, and a cold-induced activation of Δ6 and Δ5 desaturases. Choline and ethanolamine phosphoglycerides gave differential response with respect of accumulation of monounsaturated fatty acid. The level of 16:1 almost doubled in the former, and 18:1 increased by 50% in the latter. Cold-induced accumulation of oleic acid in ethanolamine phosphoglycerides has been reported in several cases (8).

About 16–20 different molecular species were identified from diacyl PE and diacyl PC, respectively (Table 2). Adaptation to winter temperatures brought about a reorganization of molecular species composition of both subclasses. 16:0/22:6, 16:0/18:1, and 18:0/22:6 (amounting to 58% of the total) were the major PC species in the SA fish. In the WA state, the level of 16:0/18:1 and 18:0/22:6 species decreased by almost 50%. 16:0/22:6 did not change at all in the course of the thermal adaptation. There was also a 75% decrease in the level of 16:0/16:0 PC. These changes were compensated for by an increase in the levels of 20:4/20:4 (75%), 18:1/20:5 (85%), 18:1/22:6 (55%), and 18:1/20:4 (52%). Altogether, the levels of 1-monounsaturated, 2-polyunsaturated species rose from 12 to 19%. 16:0/20:4 also increased in the WA fish livers. This resulted in a decrease in the ratio of the total saturated/unsaturated-to-monounsaturated/polyunsaturated PC from 6.6 to 3.3 (Table 2).

A major difference between diacyl PE and cholines from the SA and WA fish is the lower proportion of 16:0/18:1 in the latter. In general, however, the trend of the changes was similar to PC, but in addition to 18:0/22:6 and 16:0/18:1, levels of 16:0/22:6 and 18:0/20:4 also decreased from a total of 63.6 to 37.7%. There was a substantial increase in the levels of 1-monounsaturated, 2-polyunsaturated species from a total of 10.2 to 28.5%, with a concomitant 75% decrease in the

TABLE 2
Molecular Species Composition of Liver Diacyl Phosphatidylcholines and Diacyl Phosphatidylethanolamines of Carp Adapted to Summer and Winter Temperatures[a]

	Phosphatidylcholine (wt%)		Phosphatidylethanolamine (wt%)	
Species[b]	Summer	Winter	Summer	Winter
20:4/20:4	1.7	7.2	0.7	2.0
18:1/20:5	0.7	4.7	+[c]	0.9
16:1/16:1	+	+	+	0.5
18:1/22:6	3.9	6.9	5.4	10.2
16:0/22:6	22.9	23.5	23.1	15.8
16:0/22:5	0.3	1.1	+	1.8
18:1/20:4	4.3	8.2	4.8	17.4
16:0/20:4	7.7	11.4	10.0	15.9
18:1/18:2	0.6	0.6	—[d]	—
16:0/18:2	6.6	9.1	2.7	3.4
18:0/22:6	12.4	6.7	17.0	6.2
14:0/16:0	0.4	+	+	1.3
18:0/20:5	1.5	+	3.0	4.3
18:0/20:4	2.3	1.9	23.5	5.7
18:1/18:1	2.8	3.0	+	0.6
16:0/18:1	23.2	11.8	3.4	1.1
16:0/16:0	3.7	0.8	1.4	0.3
18:1/20:1	0.2	+	+	+
18:0/18:1	3.1	1.7	1.2	+
16:0/18:0	0.3	+	0.8	+
Sat[e]/sat	4.4	0.8	2.2	1.6
Sat/unsat[f]	64.2	65.5	82.7	64.2
Monoen/polyen[g]	9.6	19.8	10.2	28.5
Polyen/polyen	1.7	7.2	0.7	2.8

[a]Molecular species of the phospholipid classes were analyzed by high-performance liquid chromatography as described in the Materials and Methods section.
[b]The first number denotes the carbon chain length and the second the number of double bonds in the aliphatic chain.
[c]Traces.
[d]Not detectable.
[e]Saturated fatty acid.
[f]Unsaturated fatty acid.
[g]Monounsaturated fatty acid/polyunsaturated fatty acid.

ratio of total saturated/unsaturated-to-total monounsaturated/polyunsaturated species from 8.1 to 2.2. Similarly, in PC, there was an increase also in the level of 16:0/20:4.

These differences in molecular composition of the major phospholipids are reflected in physical properties of phospholipid vesicles probed using ESR spectroscopic technique. Figure 1 compares the order parameters between −30C and 30°C of the phospholipid vesicles made from cold- and warm-adapted fish livers. As seen, there is a change of slope, representing a thermotropic phase transition from solid gel to a more fluid liquid-crystalline state, around −13 and −8°C in the case of probes from WA and SA fish, respectively. Thus, a reduction of 20°C in the ambient temperature brought about a 5°C downshift in the thermotropic phase transition temperature.

In another approach, steady-state fluorescence polarization, R_{ss}, of different anthroyloxy fatty acids (2-AS, 12-AS, and 16-AP), reporting the corresponding membrane segments, was determined. Figure 2 shows that the phospholipid vesicles made from livers of WA fish exhibited lower R_{ss} values, that is, they were more "fluid" at all segments, than those from the SA fish. Equal anisotropy values were measured in the C_2 segment at 5°C for the vesicles from the WA fish and at 25°C for those from the SA fish.

In further experiments, anisotropy parameters of vesicles made from isolated PC were compared. Again, PC vesicles from WA carp proved to be less ordered than those from SA carp (Fig. 3), but they were less ordered at almost each segment than the corresponding total phospholipids.

In an attempt to understand the role of phospholipid molecular species showing changes during thermal adaptation, model experiments on vesicles from synthetic phospholipids also were carried out using ESR spectroscopy and 5-SASL. First it seemed logical to decide whether the downshift of the transition temperature and the increased disorder seen with vesicles made either from total phospholipids or PC of WA fish can be related to the observed alterations in molecular

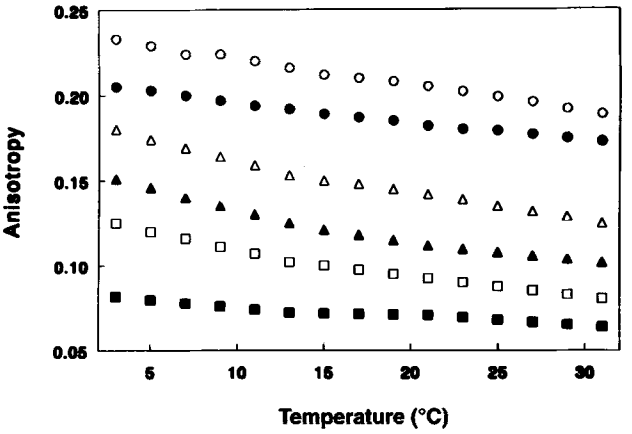


FIG. 2. Temperature dependency of fluorescence anisotropy of 2-(*N*-9-anthroloxy)stearic acid (2-AS), 12-(*N*-9-anthroyloxy)stearic acid (12-AS), and 16-(*N*-9-anthroyloxy)palmitic acid (16-AP) embedded in vesicles made from liver total phospholipids of carp adapted to summer (○, 2-AS; △, 12-AS; and □,16-AP) and winter (●, 2-AS; ▲, 12-AS; and ■,16-AP) temperatures.

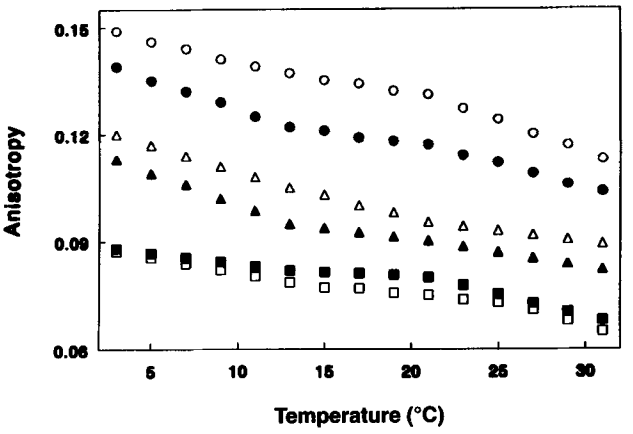


FIG. 3. Temperature dependency of fluorescence anisotropy of 2-AS, 12-AS, and 16-AP embedded in phosphatidylcholine vesicles made from livers of carp adapted to summer (●, 2-AS; ▲, 12-AS; and □, 16-AP) and winter (●, 2-AS; ▲, 12-AS; and ■, 16-AP) temperatures. Abbreviations as in Figure 2.

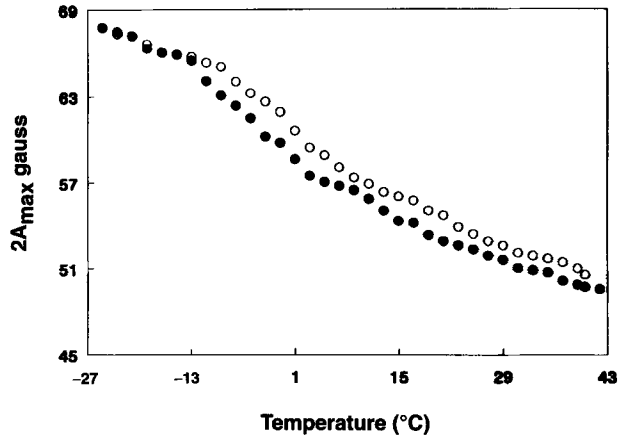


FIG. 1. Temperature dependency of outer hyperfine splitting, (2 A_{max}) of 5-doxylstearic acid in hydrated phospholipids obtained from livers of carp adapted to summer (○) and winter (●) temperatures.

species composition of PC and PE. Based on the data of Table 2, Figure 4 compares the melting temperature of 18:0/22:6 PC (100%) with ternary mixtures of synthetic PC. Vesicles made of 4% 16:0/16:0, 1% 20:4/20:4, and 94% 18:0/22:6 PC were selected to mimic the PC from SA fish, while vesicles made of 1% 16:0/16:0, 10% 20:4/20:4, and 89% 18:0/22:6 PC were selected to mimic the PC from WA fish. As seen in Figure 4, addition of 4% 16:0/16:0 PC along with 1% 20:4/20:4 PC did not significantly affect the transition temperature of 18:0/22:6 (100%) PC (−8.8 vs. −8.4°C). Increasing the amount of 20:4/20:4 PC with a concomitant reduction of the amount of 16:0/16:0 PC (from 2 to 10%, and from 4 to 1%, respectively) brought about an increase of this temperature from −8.4 to −6.2°C. The acyl chain order, reflected by the hyperfine splitting values, remained virtually unaffected in these combinations of PC.

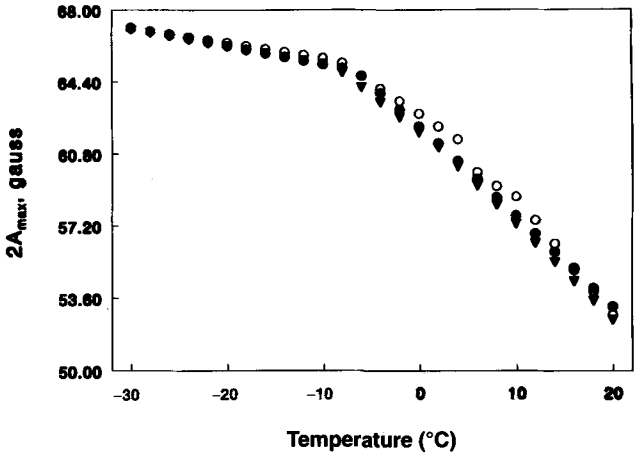


FIG. 4. Thermotropic phase transition temperature of 18:0/22:6 vesicles (▼) or in mixed phospholipid vesicles consisted of either 1% 16:0/16:0, 10% 20:4/20:4, and 89% 18:0/22:6 (●); or in 4% 16:0/16:0, 2% 20:4/20:4, and 94% 18:0/22:6 (○).

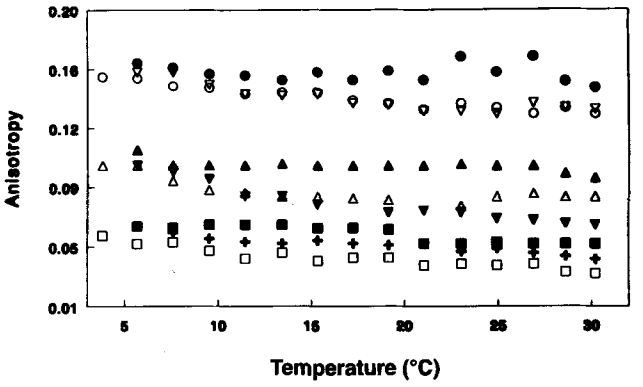


FIG. 5. Temperature dependency of fluorescence anisotropy of 2-AS, 12-AS, and 16-AP in 16:0/18:1 (▼, 2-AS; ▼, 12-AS; and +, 16-AP), 18:0/22:6 (○, 2-AS; △,12-AS; and □, 16-AP), and 22:6/22:6 (●, 2-AS; ▲, 12-AS; and ■, 16-AP) phosphatidylcholine vesicles. Abbreviations as in Figure 2.

In another series of experiments, fluorescence spectroscopy was used and anisotropy parameters of 16:0/18:1, 16:0/22:6, and 22:6/22:6 PC using 2-AS, 12-AS, and 16-AP were compared. Figure 5, in agreement with the data presented in Figure 4, shows that the R_{ss} values of these phospholipids were very close in the corresponding membrane segments. That is, introduction of 11 double bonds in PC molecules relative to 16:0/18:1 does not render the vesicles more disordered than a single Δ9 *cis* double bond in the *sn*-1 position. On the contrary, 22:6/22:6 PC were slightly more ordered at higher temperatures (above 25°C).

PC make 40–50% of the total phospholipids in the membranes (19–21), and thus, it can be expected that physical properties of these phospholipids, given the matrix of membranes, remain unaffected during thermal adaptation. Consequently, some other membrane lipid components might be involved in controlling acyl chain order in response of temperature changes. PE are the second most abundant phospholipids in membranes (19–21), comprising 20–30% of the total. As the total amounts of saturated/unsaturated and monounsaturated/polyunsaturated species showed the most characteristic changes in thermally adapted fish (Table 2), model experiments with binary mixtures of the representative molecular species of PE (16:0/18:1 and 18:1/22:6) and PC (18:0/22:6) were carried out using either ESR spectroscopy or fluorescence polarization techniques. In Figure 6, the thermotropic phase transition temperature of mixed vesicles consisting of either 18:0/22:6 PC (75%) and 18:1/22:6 PE (25%) or 18:0/22:6 PC (75%) and 16:0/18:1 PE is compared with 18:0/22:6 (100%) vesicles using ESR spectroscopy. It is evident that the presence of 18:1/22:6 PE in the model system reduced the transition temperature from −8.8 to −11°C. The acyl chain disorder also decreased, especially at lower temperatures. On the contrary, the presence of 16:0/18:1 shifted the phase transition to higher temperatures, from −8.8 to −3°C.

Figures 7 and 8 confirm and extend the observations

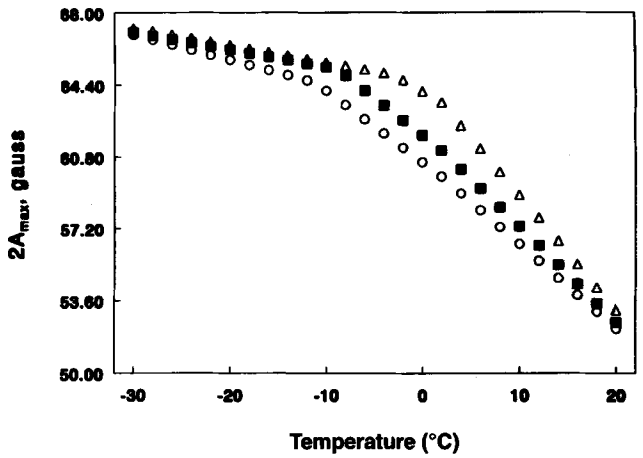


FIG. 6. Thermotropic phase transition temperatures of 18:0/22:6 phosphatidylcholine, 100% (■), 16:0/18:1 phosphatidylethanolamine, 25%; +18:0/22:6 phosphatidylcholine, 75% (▲); and 18:1/22:6 phosphatidylethanolamine, 25%; +18:0/22:6 phosphatidylcholine, 75% (●).

shown in Figure 6, that increased acyl chain disorder in the vesicles from WA fish is due to the observed accumulation of 1-monounsaturated, 2-polyunsaturated PE, and also PC species. In the model experiment shown in Figure 7, mixed vesicles consisting of 75% 16:0/22:6 PC and either 25% 16:0/18:1 (Fig. 7A) or 25% 18:1/22:6 PE (Fig. 7B) were tested. While 16:0/18:1 PE rendered the mixed vesicles more ordered in the uppermost segment, the 18:1/22:6 species increased the disorder (Fig. 7B) in comparison to the 16:0/22:6 (100%) vesicles. In the deeper layers, the ordering effect of 16:0/18:1 PE was reversed, and a slight fluidization was observed (Fig. 7A). The fact that 16:0/18:1 PE made the mixed vesicles more ordered in the C-2 segment explains the differences in anisotropy parameters of total phospholipids and purified PC (Fig. 2 vs. Fig. 3). This ordering effect of PE already has been described (22), and is attributed to interactions be-

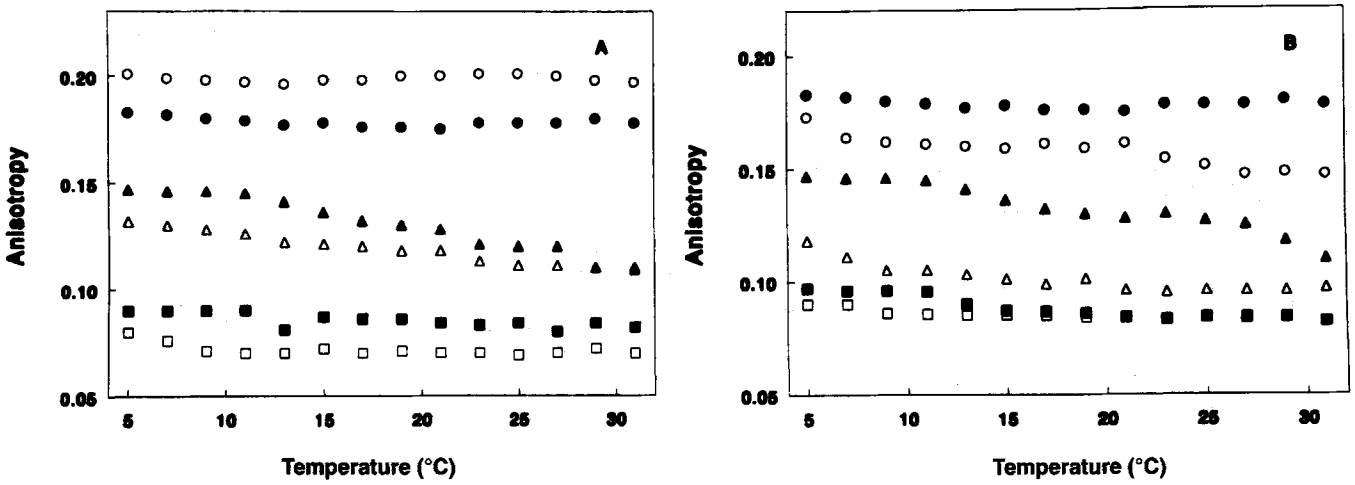


FIG. 7. Temperature dependency of fluorescence anisotropy of 2-AS, 12-AS, and 16-AP embedded either in A: 100% 16:0/22:6 phosphatidylcholine (●, 2-AS; ▲, 12-AS; and ■, 16-AP) or 75% 16:0/22:6 phosphatidylcholine and 25% 16:0/18:1 phosphatidylethanolamine (○, 2-AS; △, 12-AS; and □, 16-AP) or in B: 100% phosphatidylcholine (●, 2-AS; ▲, 12-AS; and ■, 16-AP) or 75% 16:0/22:6 phosphatidylcholine and 25% 18:1/22:6 phosphatidylethanolamine (○, 2-AS; △, 12-AS; and □, 16-AP). Abbreviations as in Figure 2.

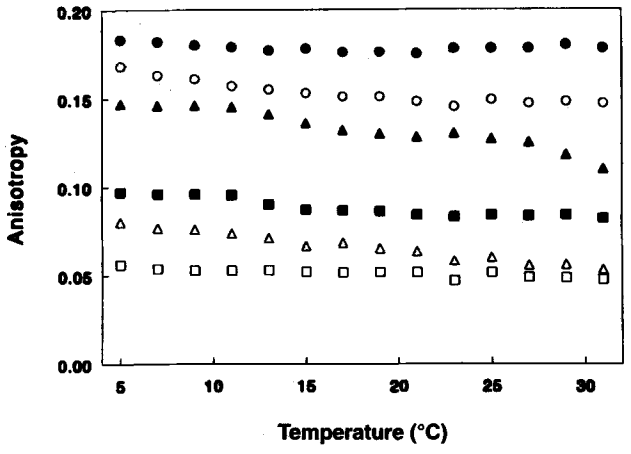


FIG. 8. Comparison of fluorescence anisotropy of 2-AS, 12-AS, and 16 AP embedded either in 100% 16:0/22:6 (●, 2-AS; ▲, 12-AS; and ■, 16-AP) or 100% 18:1/22:6 phosphatidylcholine (○, 2-AS, △, 12-AS; and □, 16-AP). Abbreviations as in Figure 2.

tween the positively charged amino N and the negatively charged phosphate ester groups of the adjacent phospholipid molecules. The presence of a *cis* double bond in the *sn*-1 position of PE may overcome these interactions with resulting increase of disorder in the hydrophobic region of the bilayer. In fact, mixing 18:1/22:6 PE with 16:0/22:6 PC resulted in a drastic fluidization in both the C_2 and C_{12} segment that is just beyond the position of the olefinic bond of oleic acid. A similar picture is seen when anisotropy parameters of 18:1/22:6 and 16:0/22:6 PC are compared, with the exception that vesicles made from 18:1/22:6 PC were more fluid in the C-16 segment than its 16:0/22:6 analog (Fig. 8). Using these model systems, the temperature-related changes in structural order of fatty acyl chains in phospholipid vesicles could be successfully modelled.

DISCUSSION

Besides temperature, the photoperiod, nutritional, and hormonal state of fish may affect metabolism of lipids and, hence, the composition of biomembranes. Although several lines of evidence show a compensation of the membrane ordering state for changes in the ambient temperature (23–27), little information is available about the alterations taking place at the structural level within the bilayer during thermal adaptation. It is also unclear how the individual phospholipid molecules participate in this process. The results presented in Figure 1 clearly show that the transition from a solid gel to a melted, liquid-crystalline state is shifted down by about 5°C in phospholipids in the livers of WA carps. This, and comparison of hyperfine splitting values of phospholipids from WA fish at 5°C and from warm-adapted fish at 11°C, indicate a roughly 25–30% compensation of the membrane ordering state for the temperature. Using fluorescent probes and a different approach, this compensation is 100% in each segment scanned.

Both the downshift of the transition temperature and the increase of the acyl chain disorder in the bilayer might be related to the observed changes in the molecular species composition of the major phospholipids, diacyl PC, and PE. The most important changes in molecular species composition of these phospholipids are a decrease in the level of 18:0/22:6 species and a concomitant increase in the levels of 1-monounsaturated, 2-polyunsaturated (18:1/22:6, 18:1/20:4) species during adaptation to winter temperatures (Table 2). Accumulation of 18:1/22:6 and, to some extent, 18:1/20:5 PE, also has been demonstrated for marine fish and shrimp inhabiting polar and subtropic waters (8,9) or brains of freshwater fish adapted to reduced temperatures (10). The thermotropic phase transition temperatures of 18:0/22:6 PC and 16:0/22:6 PC are rather close. Thus, accumulation of 16:0/22:6 PC to the cost of 18:0/22:6 species (Table 2) might not have been

responsible for the observed downshift of the thermotropic phase transition temperature of phospholipid vesicles. Moreover, model experiments made on vesicles of PC consisting of disaturated (having high phase transition temperatures, above 40°C) and polyunsaturated (having extremely low phase transition temperatures, below −40°C), as well as saturated/polyunsaturated PC species (having intermittent phase transition temperatures, around −10°C), revealed that these species in concentrations recorded in cold/warm-adapted livers (Table 2), do not participate in the control of membrane physical properties according to the temperature. Thus, such changes alone do not explain either the increased disorder in the fatty acyl chains or the downshift of the thermotropic phase transition temperature in the WA state. The noninvolvement of polyunsaturated fatty acids/phospholipid molecular species in controlling membrane physical state also is supported by the results of Stubbs and Kisielewski (28) on sarcoplasmic reticulum of rats fed either fish oil or vegetable oil. Despite the increase in the levels of long-chain polyunsaturated fatty acids in membranes of fish oil-fed group, the acyl chain disorder remained unchanged measured using diphenylhexatrione and trimethylammonium–diphenylhexatrione. Our study also did not reveal any effect of long-chain polyunsaturated fatty acids in the C_2, C_{12}, or C_{16} region of the vesicles made from livers of fish oil-fed rats compared to the vegetable oil-fed rats (9).

A characteristic difference between carp and rat liver phospholipids is the absence and cold-induced accumulation of 18:1/22:6 and 18:1/20:5 PE and PE in the latter. Figures 6, 7, and 8 clearly suggest a drastic disordering effect of 18:1/22:6 PE (Fig. 7B) and choline species (Fig. 8) in each segment of the bilayer. Previously we have shown that combination of rat liver PC (75%) with salmon liver PE (25%) rich in 18:1/20:5 and 18:1/22:6 species resulted in a considerable fluidization, compared with vesicles made from salmon liver PC (75%) and rat liver PE (25%) (9). In addition, 18:1/22:6 PE in contrast to 16:0/18:1 PE, shifts the thermotropic phase transition to lower temperatures (Fig. 7). Thus it can be proposed that increased disorder and lower transition temperatures of membranes of marine and freshwater fish, either evolutionary or seasonally-adapted to reduced temperatures, are at least partially due to an increase in the levels of 1-monounsaturated, 2-polyunsaturated PE species.

However, the technique used to incorporate the labels into the vesicles does not permit the differentiation between the outer and inner membrane leaflet. But, because PE are conic-shaped molecules (29), it is reasonable to speculate that most of them, similar to the native membranes, are enriched in the inner leaflet of the membrane to ensure the curvature of these structures. Considering this, the increase of fluidity in the inner leaflet of vesicles from the WA fish might have been considerable. However, native membranes might differ considerably in this respect and the fluidizing effect of 1-monoenoic, 2-polyenic PE species might be influenced by the presence of proteins and other membrane components. Increased disorder in the outer leaflet of the bilayer can be explained by accumulation of molecules like 18:1/22:6 PC. This can be an explanation of why PC vesicles from cold-adapted carps were more disordered than those from the warm-adapted specimens, and why the outer leaflet of hepatocyte plasma membranes of cold-exposed trout become more fluid than those of warm-exposed trout (30). Based on this, it can be proposed that "fine tuning" of the ratio of 1-saturated, 2-unsaturated, and that of the 1-monounsaturated, 2-polyunsaturated PE might be an important factor determining the molecular architecture of membranes in relation to thermal adaptation. However, we are aware that these experiments are oversimplifications of interactions taking place in native membranes at molecular and structural level because there are a large number of phospholipids present not considered in this study, in addition to cholesterol and proteins. Although more investigations are necessary to prove this, it has become evident from the results presented here that not the gross amounts of long-chain polyunsaturated fatty acids, but their specific pairing with monounsaturated fatty acids in the *sn-1* position might be an important factor in adjusting membrane architecture and physical properties to temperature in fish.

ACKNOWLEDGMENTS

This paper has been supported by an OTKA grant to T.F. (grant No T 5163). Thanks also go to the Tisza Fish Farm for supplying fish for the experiments.

REFERENCES

1. Singer, S.J., and Nicholson, G.L. (1972) The Fluid Mosaic Model of the Structure of Cell Membranes, *Science 175*, 720–731.
2. Sinensky, M. (1974) Homeoviscous Adaptation—A Homeostatic Process that Regulates the Viscosity of Membrane Lipids in *Escherichia coli*, *Proc. Natl. Acad. Sci., USA. 71*, 522–525.
3. Hazel, J.R., and Prosser, C.L. (1974) Molecular Mechanisms of Temperature Compensation in Poikilotherms, *Physiol. Rev. 54*, 620–627.
4. Williams, E.E., and Hazel, J.R. (1992) The Role of Docosahexaenoic Acid-Containing Molecular Species of Phospholipid in the Thermal Adaption of Biological Membranes (Sinclair, A., and Gibsson, R., eds.) Champaign, pp. 128–132.
5. Wallrecht, C.H., and Babin, P.J. (1994) Thermal Adaption Affects the Fatty Acid Composition of Plasma Phospholipids in Trout, *Lipids 29*, 373–376.
6. Coolebar, K.P. Beroe, C.B., and Keough, K.M.W. (1983) Gel to Liquid-Crystalline Phase Transitions of Aqueous Dispersions of Polyunsatruated Mixed-Acid Phosphatidylcholines, *Biochemistry 22*, 1466–1473.
7. Evans, R.W., Williams, M., and Tinoco, J. (1987) Surface Areas of 1-Palmitoyl Phosphatidylcholines and Their Interactions with Cholesterol [published erratum appears in *Biochem J.* (1987); *246*: 806] *Biochem. J. 245*, 455–467.
8. Dey, I., Buda, Cs., Viik, T., Halver, J.A., and Farkas, T. (1993) Molecular and Structural Composition of Phospholipid Membranes in Livers of Marine and Freshwater Fish in Relation to Temperature, *Proc. Natl. Acad. Sci. USA. 90*, 7498–7502.
9. Farkas, T., Dey, I., Buda, Cs., and Halver, J.E. (1994) Role of Phospholipid Molecular Species in Maintaining Lipid Membrane Structure in Response to Temperature, *Biophys. Chem. 50*, 147–155.

10. Buda, Cs., Dey, I., Balogh, N., Horváth, L.I., Maderspach, K., Juhász, M., Yeo, Y.K., and Farkas, T. (1994) Structural Order of Membranes and Composition of Phospholipids in Fish Brain Cells During Thermal Acclimatization, *Proc. Natl. Acad. Sci. USA 91*, 8234–8238.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
12. Fine, J.B, and Sprecher, H. (1982) Unidimensional Thin-Layer Chromatography of Phospholipids on Boric Acid-Impregnated Plates, *J. Lipid. Res. 23*, 660–663.
13. Takamura, H., Narita, M., Urade, R., and Kito, M. (1986) Quantitative Analysis of Polyenoic Phospholipid Molecular Species by High-Performance Liquid Chromatography, *Lipids 21*, 356–361.
14. Bell, M.V., and Dick, J.R. (1991) Molecular Species Composition of the Major Diacyl Glycerophospholipids from Muscles, Liver, Retina and Brain of Cod (*Gadus morhua*), *Lipids 26*, 565–573.
15. Sen, A., Thomas, V., Isac, T.V., and Hui, S.W. (1991) Bilayer Packing Stress and Defects in Mixed Dilinoleoylphosphatidylethanolamine and Palmitoyloleoylphosphatidylcholine and Their Susceptibility to Phospholipase A_2, *Biochemistry 30*, 4516–4524.
16. Freed, J.H. (1976) Theory of Slow Tumbling ESR Spectra for Nitroxydes, in *Spin Labeling, Theory and Applications* (Berliner, L.J., ed.) Academic Press, New York, London, pp. 53–133.
17. Jones, R.H., and Dey, I. (1995) Determining One or More Change Points, *Chem. and Phs. of Lipids 76*, 1–6.
18. Farkas, T., and Roy, R. (1989) Temperature Mediated Restructuring of Phosphatidylethanolanines in Livers of Freshwater Fishes, *Comp. Biochem. Physiol. 79B93*, 217–222.
19. Leslie, J.M., and Buckley, J.T. (1976) Phospholipid Composition of Goldfish (*Charassius auratus* L.) Liver and Brain and Temperature-Dependence of Phosphatidylcholine Synthesis, *Comp. Biochem. Physiol. 53B*, 335–357.
20. Wodtke, E. (1978) Lipid Adaption in Liver Mitochondrial Membranes of Carp Acclimated to Different Environmental Temperatures: Phospholipid Composition, Fatty Acid Pattern and Cholesterol Content, *Biochim. Biophys. Acta 529*, 280–291.
21. Thillart, G. van den, and Bruin, G., de (1981) Influence of Environmental Temperature on Mitochondrial Membranes, *Biochim. Biophys. Acta 640*, 439–447.
22. Michaelson, D.M., Horwitz, A.F., and Klein, M.P. (1974) Head Group Modulation of Membrane Fluidity in Sonicated Phospholipid Dispersions, *Biochemistry 13*, 2605–2612.
23. Cossins, A.R., and Prosser, C.L. (1978) Evolutionary Adaptation of Membranes to Temperature, *Proc. Natl. Acad. Sci. USA. 75*, 2040–2043.
24 Lee, J.A.C., and Cossins, A.R. (1990) Temperature Adaption of Biological Membranes: Differential Homeoviscous Responses in Brush-Border and Basolateral Membranes of Carp Intestinal Mucosa, *Biochim. Biophys. Acta 1026*, 195–203.
25. Cossins, A.R., Kent, J., and Prosser, C.L. (1980) A Steady State and Differential Polarized Phase Fluorimetric Study of the Liver Microsomal and Mitochondrial Membranes of Thermally Acclimated Green Sunfish (*Lepomis cyanellus*), *Biochim. Biophys. Acta 599*, 341–358.
26. Lagerspetz, K.Y.H., and Laine, A. (1984) Fluidity of Epidermal Cell Membranes and Thermal Acclimation of Na_+ Transport in Frog Skin, *Mol. Physiol. 6*, 211–220.
27. Abbruzzini, A.F., Ingram, L.O., and Clem, W. (1982) Temperature-Mediated Processes in Teleost Immunity: Homeoviscous Adaptation in Teleost Lymphocytes, *Proc. Soc. Exp. Biol. Med. 169*, 12–18.
28. Stubbs, C., and Kisielewski, A.E. (1990) Effect of Increasing the Level of ω3 Fatty Acids on Rat Skeletal Muscle Sarcoplasm with Reticulum, *Lipids 25*, 553–558.
29. Cullis, P.R., and De Kruijf, B. (1979) Lipid Polymorphism and the Functional Roles of Lipids in Biological Membranes [Review], *Biochim. Biophys. Acta 559*, 399–420.
30. Williams E.E., and Hazel, J.R. (1994) Membrane Fluidity and Hemilayer Temperature Sensitivity in Trout Hepatocytes During Brief *in vitro* Cold Exposure, *Am. J. Physiol. 266*, R773–R780.

[Received February 21, 1995, and in final revised form October 2, 1995; Revision accepted October 26, 1995]

Composition of Phospholipids of White Muscle of Six Tuna Species

Isabel Medina*, Santiago P. Aubourg, and Ricardo Pérez Martín

Instituto de Investigaciones Marinas del CSIC, E-36208 Vigo, Spain

ABSTRACT: A comparative study of the phospholipids of white muscle of six of the commercially utilized tuna species, including quantitative analyses of phospholipid classes and studies of the acyl composition of the major components. Plasmalogen compounds also were identified and quantified. Choline and ethanolamine glycerophospholipids were the most abundant classes in all the samples, as well as the only molecules containing plasmalogens (16:0, 18:0, and 18:1 alkenyl-ether chains). The patterns of fatty acid distribution within each of the phospholipid classes showed general similarities in the species analyzed. However, ratios between certain saturated and polyunsaturated fatty acids in different phospholipid classes showed remarkable differences. The high content of n-3 polyunsaturated fatty acids in the principal phospholipids, such as the plasmalogens, and taking into account the fatty acids possible importance in human nutrition, indicates that the white muscle of tuna species may be a potentially important dietary item.

Lipids 30, 1127–1135 (1995).

Tuna processing is an industry of well-known economic importance in many countries, including Spain, supporting a significant market demand and playing an important role in the field of human nutrition as components of the Spanish diet (1). One of the most important aspects in the quality of marine foods concerns their lipid content and composition. Marine lipids are known for their highly unsaturated fatty acid composition (2,3), which makes them very prone to oxidative damage (4,5). On the other hand, these lipids are now the subject of a great deal of attention due to their high content of n-3 polyunsaturated fatty acids (PUFA), which have potential roles to play against certain diseases (6,7).

Several papers describing the lipid compositions of tuna fish and their alterations during frozen storage or thermal processing have been published (8–10). Most of the work carried out has been focused on the composition of the simpler lipid classes containing two types of primary products per mole, such as triacylglycerides (11–13), and literature data related to complex lipids (classes that contain three or more different primary products per mole), such as phospholipids (PL) and, especially, plasmalogens (PLA), are quite limited (14–16).

The PL composition of fish species that are consumed as foods has attracted a great deal of attention due to several characteristics: (i) they possess a highly unsaturated fatty acid composition which makes them especially vulnerable to damages during processing; (ii) some of the PL classes have been reported to possess antioxidant properties (17); and (iii) in contrast with triacylglycerides, the distribution of the PL classes with respect to their fatty acid composition is little influenced by external and internal factors, an aspect that may be relevant for species characterization purposes (18–21). Further, the abundance in n-3 PUFA of these PL may give the fish muscle a supplementary nutritional value.

The aim of the present work was to study the PL composition in several of the tuna species most widely consumed in Spain prior to processing. Different chromatographic techniques, such as high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and gas chromatography (GC) have been combined to complete PL class identifications and quantification. The compositions of fatty acids and alkenyl ethers on the glycerol moieties were studied simultaneously using an acid derivatization procedure and subsequent analysis of the corresponding methyl esters and dimethylacetals (DMA) by GC. For identification purposes, GC coupled with mass spectrometry was employed.

MATERIALS AND METHODS

Raw material and lipid extraction. Six commercial tuna species were employed—big eye tuna (*Thunnus obesus*), bluefin (*T. thynnus*), bonito (*Sarda sarda*), frigate (*Auxis thazard*), skipjack (*Katsuwonus pelamis*), and yellowfin (*T. albacares*). The fish came from the Atlantic Ocean and were purchased at a commercial market. All fish except bonito were landed frozen. Bonito fish were caught and kept on ice for three days. After arrival at our laboratory, the bonito were frozen at −40°C, and all samples were stored at −20°C prior to analysis. Five individual fish for each species were used.

Fish were skinned, beheaded, and eviscerated, and the red muscle was removed. Lipids were extracted from the white muscle by the Bligh and Dyer method (22), and the muscle

*To whom correspondence should be addressed at Instituto de Investigaciones Marinas del CSIC, Eduardo Cabello 6, E-36208 Vigo, Spain.

Abbreviations: DMA, dimethylacetal; FAME, fatty acid methyl ester; GC, gas chromatography; HPLC, high-performance liquid chromatography; LPC, lysophosphaditylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLA, plasmalogen; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SPH; sphingomyelin; TLC, thin-layer chromatography.

Copyright © 1995 by AOCS Press

content was determined gravimetrically as described by Herbes and Allen (23). All organic solvents employed were reagent grade (E. Merck, Darmstadt, Germany).

Free fatty acid determination. Free fatty acids were measured using the Lowry and Tinsley (24) method based on the formation of a complex with $(AcO)_2Cu$–pyridine (E. Merck).

Determination and purification of PL. Organic phosphorus was determined on total lipid extracts according to the Raheja *et al.* method (25) based on a complex formation with ammonium molybdate (E. Merck). Lipid extracts were subjected to TLC on 20 × 20 cm glass plates coated with Silica Gel 60W (0.8 mm) (E. Merck) and were developed twice in the same direction with $CHCl_3/CH_3OH/CH_3COOH$ (100:15:2, by vol). The polar fraction remained at the bottom and was recovered from the silica gel by elution with $CHCl_3/CH_3OH$ (2:1, vol/vol).

Fractionation of PL classes. Total PL extracts were resolved by high-performance TLC on 10 × 10 cm glass plates coated with silica gel 60W (0.25 mm) (E. Merck) and developed twice in the same direction in a solvent system of chloroform/methanol/ethyl acetate/isopropanol/KCl (0.25%) (30:9:25:6:18, by vol) (26). KCl and all organic solvents were reagent grade (E. Merck). Spots were visualized after spraying with cupric sulfate and subsequent heating at 160°C for 10 min. Individual PL classes were identified by comparison with PL standards (Matreya Inc., Pleasant Gap, PA). Optical densities of the charred spots were measured at 360 nm with a Shimadzu CS-910 dual-wavelength TLC scanner (Kyoto, Japan). In this way, sphingomyelin (SPH) and other minor PL classes, characterized by an essential saturated fatty acid composition, were quantified. Quantitation of other PL classes was not possible following this procedure as they showed highly unsaturated fatty acid composition, whereas the composition of the PL standards used was largely saturated (27). To determine the PLA content in choline and ethanolamine glycerophospholipids, elution on TLC plates (Silica Gel 60W, 20 × 20 cm, 0.25 mm) using the procedure described previously was performed. Spots corresponding to these classes, combining the diacyl and PLA forms [phosphatidylcholine (PC)–PLA, phosphatidylethanolamine (PE)–PLA], were scraped off and the lipid recovered for subsequent GC analysis.

Determination of PL fatty acid composition. To obtain the fatty acid composition of individual PL, a combination of HPLC and GC techniques was carried out. HPLC was performed with a Silica Gel 60 column (4.6 mm i.d × 25 cm.; Supelco, Inc., Bellefonte, PA), coupled with a Perkin Elmer Solvent Pump (Beaconsfield, Buckinghamshire, United Kingdom), a Perkin Elmer LC-65 UV variable wavelength detector set at 205 nm, and a Hewlett-Packard 8380A integrator (Wotingham, Berkshire, United Kingdom). The separation was achieved with an isocratic elution in a solvent mixture of $CH_3CN/CH_3OH/H_3PO_4$ (98:1:1, by vol). The flow rate was maintained at 1 mL/min. All solvents employed were of HPLC grade (E. Merck).

Polar lipids (150 μg) were injected into the HPLC system, and the PL classes were separated under the conditions described previously. The identity of peaks was verified using PL standards. Diacyl forms of phosphatidylinositol (PI), phosphatidylserine (PS), PE, PC, and lysophosphatidylcholine (LPC) were separated completely, whereas other minor complex classes and PLA eluted with the eluent front. As a result of this separation, purified diacyl forms corresponding to the PC, PE, PI, PS classes and the monoacyl LPC class were obtained, and were transmethylated to determine their acyl composition. To eliminate the excess of H_3PO_4 prior to the transmethylation, a washing step with an alkaline solution (6 % K_2CO_3) (E. Merck) was necessary.

Transmethylation of lipids. Purified diacyl PL classes coming from the above HPLC separation, and spots scraped from TLC plates containing both diacyl and alkenyl–acyl molecular species were converted into the corresponding fatty acid methyl esters (FAME) and DMA by the method of Lepage and Roy (28).

Quantification of PL classes. Transmethylated lipids containing mixtures of FAME and DMA were analyzed simultaneously by GC (Perkin Elmer 8700 chromatograph) employing a fused silica capillary column SP-2330 (0.25 mm i. d. × 30 m; Supelco Inc.), programmed from 145 to 190°C at 1.0°C/min, from 190 to 210°C at 5.0°C/min, and then followed by a hold for 13.5 min at 210°C. Nitrogen at 10 psig was the carrier gas, and a flame-ionization detector set at 250°C was used. A programmed temperature injector was used in the split mode (150:1) and heated from 45 to 275°C at 15°C/min.

Peaks were identified by comparison of their retention times with standard FAME mixtures (Supelco: PUFA No. 1 and No. 2; Larodan, Qualmix Fish). For quantitation purposes, peak areas were automatically integrated, and 19:0 fatty acid (Sigma, St. Louis, MO) was used as an internal standard.

Quantification of diacyl PL classes (PE, PC, PS, PI) and the monoacyl LPC was done knowing their respective fatty acid composition and the structure of the nonacyl moiety. The PLA content was obtained by doubling the molar percentage of total DMA in the mixture of DMA and FAME obtained after transmethylation of polar lipids, according to Visnaanathan *et al.* (29). Total contents corresponding to PC and PE were determined by combining the quantities of diacyl and PLA forms.

GC/mass spectrometry. A Hewlett-Packard model 5890 A chromatograph, equipped with on-column capillary injection, was used with a 5970 series mass selective detector. The separation was made using the same column and conditions described previously. Helium was used as carrier gas. The mass spectrometer was operated in the electron impact ionization mode (70 eV). The transfer line and ion source temperature were both 280°C. Mass spectra of the different peaks were checked in order to differentiate FAME from DMA. The structures of the DMA components were investigated.

RESULTS AND DISCUSSION

Lipid and PL content. The lipid content for each species was rather constant, as can be observed from the low standard deviations calculated for five individual samples (Table 1).

TABLE 1
Lipid and Phospholipid Contents[a] in White Muscle of Six Tuna Species

Tuna species	Lipid[b]	Phospholipid[c]
Bigeye	0.76 ± 0.29	35.78 ± 6.99
Bluefin	0.95 ± 0.48	26.41 ± 5.30
Bonito	1.94 ± 0.19	1.60 ± 0.16
Frigate	0.65 ± 0.20	37.81 ± 5.86
Skipjack	0.78 ± 0.22	28.54 ± 7.52
Yellowfin	0.57 ± 0.04	58.80 ± 7.56

[a]Results are expressed as mean ± standard deviation for five samples.
[b]Expressed as g/100 g wet muscle.
[c]Expressed as percent of total lipids.

Specimens corresponding to the bonito species showed the highest lipid content with results which confirmed those obtained for the other species. A great variability in the lipid content has been observed in migrating fish species, depending on both enviromental and endogenus factors (12,30–32).

On the other hand, possible decomposition of lipids during frozen storage of samples prior their acquisition also must be also considered (33,34). In order to clarify the extent of this reaction, the amounts of free fatty acids in all samples were calculated. Levels of free fatty acids were generally low, with values ranging between 0–5% of total lipid for all samples, the lowest values being those for bonito. The extent of lipid hydrolysis thus could not explain the low lipid content in respect to the bonito samples. Therefore, the lipid contents found must be considered as completely natural in these tunas, and data published for tuna species, such as skipjack or yellowfin (35,36), were very similar to those determined in this work.

All species except bonito showed high percentages of PL (Table 1). Because PL contain more of the highly unsaturated fatty acids than do triacylglycerols, the white muscle of these species could be considered an important dietary source of PUFA.

PL content in muscle has proven to be relatively constant according to their presence in cellular membranes (3); this fact has led to the hypothesis of an inverse ratio between lipid content and PL proportion in total lipids (37). The regression between the lipid and the PL content in the present study has demonstrated an inverse correlation (correlation coefficient = −0.751) probably related to the previously mentioned stability of PL content as opposed to the significant variations that may occur in the amount of simpler lipids in migrating fish species (3).

PL classes quantification. Table 2 shows the distribution of PL classes in each species. All species analyzed showed a similar qualitative and quantitative pattern of PL classes. In all cases, PC was the major class, followed by PE and LPC. PC and, secondly, PE usually have been measured as the main PL classes in fish species (11,38,39).

Minor classes such as phosphatidylglycerol and phosphatidic acid also could be identified. In fact, some authors have described the presence of both PL classes in very low proportions in a wide variety of fish tissues (40,41).

The levels of LPC ranged between 15–20% in all tuna species studied. Previous researchers have described the presence of lyso-PL forms in commercially available fresh tuna muscle and the increase in their content during storage at low temperatures (9,14). In fact, it has been accepted that enzymatic PL hydrolysis can occur slowly during frozen storage or in the muscle of several fish species when held on ice (42). At this point, it seems clear that a certain level of hydrolysis in PL has taken place in all samples during its storage prior to acquisition. However, the extent of that hydrolysis did not appear to be great as samples in which no free fatty acids (bonito) were detected showed proportions of LPC similar to those with higher levels (Table 2).

Other important differences have been recognized in PI and SPH contents among the species. Both PL have been found in several marine samples, although their concentrations vary depending on the tissue and organ considered (38,43).

PLA. Table 3 shows the PLA contents obtained in the different tuna species. Some papers have described the presence of alkenyl ethers in a wide variety of fish muscle (15,44). Levels of ether-linked PL in food have a particular interest from a dietary point of view, as its ingestion could contribute to the production of potent biochemical mediators (45). Further, the PLA content and composition have been shown to be relevant when studying changes in processed foods, as the content decreased as a result of thermal treatment (46). Both aspects may be important in this study, taking into account

TABLE 2
Percentage Distribution[a] of Phospholipid (PL) Classes in White Muscle of Six Tuna Species

PL class[b]	Tuna species					
	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
PE	18.81 ± 1.11	18.98 ± 1.27	20.05 ± 0.35	21.84 ± 1.98	20.18 ± 2.27	21.03 ± 1.87
PI	5.81 ± 0.86	6.71 ± 2.18	2.25 ± 0.05	10.88 ± 2.17	4.90 ± 0.70	8.46 ± 1.70
PS	5.42 ± 1.42	4.82 ± 1.27	2.20 ± 0.01	5.11 ± 1.42	5.04 ± 1.93	5.37 ± 1.17
PC	42.13 ± 3.58	42.18 ± 5.50	53.85 ± 0.35	47.36 ± 3.78	51.52 ± 5.56	37.88 ± 3.26
SPH	3.33 ± 0.96	5.55 ± 0.59	7.55 ± 0.45	2.98 ± 1.70	0.50 ± 0.04	3.98 ± 1.63
LPC	22.13 ± 3.85	15.40 ± 3.21	13.80 ± 0.40	12.02 ± 3.22	18.27 ± 0.94	21.54 ± 3.95
Others	4.41 ± 2.95	6.64 ± 0.80	trace	1.73 ± 0.50	1.52 ± 0.37	2.75 ± 0.36

[a]Results are expressed as mean ± standard deviation for five samples.
[b]PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

TABLE 3
Total Plasmalogen (PLA) Content[a] of Complex Lipids and of Phosphatidylcholine and Phosphatidylethanolamine in White Muscle of Six Tuna Species

	Tuna species					
PL Content	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
% Total	10.54 ± 2.18	15.92 ± 2.68	17.01 ± 1.40	11.93 ± 2.10	20.50 ± 2.73	13.07 ± 2.52
PC–PLA[b]	7.77 ± 1.24	12.37 ± 2.23	37.89 ± 1.18	7.80 ± 0.40	17.99 ± 2.26	6.53 ± 0.36
PE–PLA[b]	38.87 ± 5.88	41.37 ± 1.83	62.11 ± 1.18	40.51 ± 3.81	56.85 ± 3.28	49.02 ± 5.80

[a]Means and standard deviations for five samples.
[b]Results are expressed as percentage of each plasmalogen in the phospholipid class. Abbreviations as in Table 2.

TABLE 4
Alkenyl Ether Composition[a] of PE–PLA and PC–PLA in White Muscle of Six Tuna Species

	PE			PC		
Tuna species	16:0[b]	18:0[b]	18:1n-9[b]	16:0[b]	18:0[b]	18:1n-9[b]
Bigeye	46.54 ± 1.04	33.85 ± 1.99	19.61 ± 1.14	71.65 ± 2.43	17.02 ± 2.10	10.75 ± 2.10
Bluefin	65.58 ± 0.51	26.10 ± 0.77	8.32 ± 0.26	77.17 ± 1.72	33.85 ± 1.99	19.61 ± 1.14
Bonito	50.67 ± 2.04	34.14 ± 1.09	14.73 ± 1.23	66.37 ± 1.22	23.07 ± 1.25	10.56 ± 1.01
Frigate	40.03 ± 2.16	51.57 ± 1.61	8.40 ± 0.69	76.15 ± 2.25	17.88 ± 1.77	5.97 ± 0.78
Skipjack	53.22 ± 0.88	35.59 ± 0.98	11.28 ± 1.21	79.54 ± 1.49	13.98 ± 1.25	6.98 ± 0.77
Yellowfin	58.04 ± 1.62	33.30 ± 0.87	8.67 ± 0.75	78.33 ± 3.21	15.48 ± 1.04	8.67 ± 0.75

[a]Means and standard deviations for five samples. Results are expressed as percentage of each fatty chain in the *sn*-1 position of the plasmalogen moiety. Abbreviations as in Tables 2 and 3.
[b]Long-chain alkenyl ethers.

that all species have shown high levels of PLA in the white muscle (Table 3).

The PLA proportion was found to have a wide range in the tuna species studied, but a simple relationship with the PL content could not be determined (Table 1). The comparison of both parameters in most of the species studied suggests that an inverse trend with respect to the PL concentration in muscle may exist, although the low PL content found in bonito is in disagreement with this possibility. In addition, some papers have proposed a possible mechanism of adaptation to lower temperatures in marine organisms which involves a decrease in the PLA content with respect to diacyl forms of PL (44,47).

Finally, an enzymatic hydrolysis of PLA in marine invertebrates has been described during frozen storage, although it occurs more slowly than for diacylphospholipids (48). Because no significant hydrolysis seems to take place in diacyl forms as a result of frozen storage, the extent of this reaction on PLA is likely to be insignificant.

From their mass spectra, DMA were identified as a result of the methanolysis of alkenyl ether chains corresponding to 16:0, 18:0, and 18:1n-9 fatty acids in accordance with previous results determined in *Euthynnus pelamis* (15,49). Other fatty chains, such as 17:0, 20:0, and 21:0, were detected as trace components.

Alkenyl ethers were found only as PC–PLA and PE–PLA forms for all the tuna species studied (Table 3). Both major PLA usually have been found in marine tissues in association with the major PL (3,36). As can be seen in Table 3, ethanolamine glycerophospholipids showed the largest concentration in PLA, reaching values to nearly 50% of the class.

With respect to the composition of the alkenyl ether chains, two different patterns were observed for each PLA class (Table 4). PC–PLA showed an important concentration in 16:0 (approximately 75%), whereas the levels of 18:0 and 18:1n-9 in PE–PLA were higher than those found in its homologue PC–PLA. This situation has been described previously for other marine organisms (50,51).

1-*O*-Alkyl-2-acylglycerophospholipids also have been found in some tissues of some marine organisms (3,14), but they were not detected at significant levels in this research.

Fatty acid composition. Fish PL are remarkable for their high content in PUFA, and substantial differences in their fatty acid composition could be found, depending on the muscle tissue or organism (3,39). Fatty acid compositions of the five major PL classes in six tuna species are shown in Tables 5–9. SPH was a minor class for all tuna species; it also showed a less complicated fatty acid composition with 16:0 (30–35%), 18:1n-9 (23–39%), and 24:1n-9 (12–15%) as major components, and low contents of PUFA (12% of 22:6n-3 approximately). This composition is similar to that obtained by Linares and Henderson (52) in trout muscle.

As can be inferred from Tables 5–9, the fatty acid composition exhibits certain discrepancies between classes and even between species. However, very characteristic patterns could be recognized between lipid classes originating in different samples. One of the most noteworthy was the preponderance of stearic acid over palmitic acid as the principal saturated acid in PE, and the high level of palmitic acid in PC. This distribution of 16:0 and 18:0 fatty acids seems to be a characteristic of the PL from a number of tissues (53), and it is difficult to reconcile with the hypothesis that both PL come from

TABLE 5
Fatty Acid Composition[a] of Phosphatidylcholine from White Muscle of Six Tuna Species

Acid	Tuna species					
	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
14:0	1.11 ± 0.22	3.78 ± 1.06	1.12 ± 0.43	1.48 ± 0.57	1.01 ± 0.19	1.43 ± 0.24
15:0	0.50 ± 0.07	1.24 ± 0.19	0.61 ± 0.12	0.73 ± 0.12	0.53 ± 0.06	0.68 ± 0.14
16:0	20.20 ± 1.94	20.05 ± 1.94	23.22 ± 2.02	25.46 ± 0.97	21.46 ± 1.82	19.26 ± 1.20
17:0	1.19 ± 0.246	1.20 ± 0.15	1.26 ± 0.08	1.07 ± 0.18	1.07 ± 0.13	1.23 ± 0.13
18:0	3.03 ± 0.41	5.96 ± 1.44	4.19 ± 1.25	4.51 ± 0.71	3.34 ± 0.47	3.60 ± 0.40
20:0	0.35 ± 0.08	2.97 ± 2.07	0.80 ± 0.20	0.37 ± 0.13	0.00 ± 0.00	0.47 ± 0.27
24:0	0.46 ± 0.38	1.34 ± 0.22	0.47 ± 0.22	0.46 ± 0.21	0.48 ± 0.17	0.77 ± 0.24
16:1n-5	0.42 ± 0.06	1.27 ± 0.14	0.55 ± 0.27	0.41 ± 0.22	0.31 ± 0.04	0.62 ± 0.28
16:1n-7	0.79 ± 0.17	2.47 ± 0.20	0.79 ± 0.28	0.93 ± 0.19	0.81 ± 0.05	0.92 ± 0.19
16:1n-9	0.49 ± 0.05	0.34 ± 0.18	0.49 ± 0.25	0.54 ± 0.19	0.76 ± 0.08	0.55 ± 0.25
18:1n-7	1.23 ± 0.243	1.58 ± 0.19	0.97 ± 0.39	1.27 ± 0.22	1.06 ± 0.08	1.26 ± 0.07
18:1n-9	10.49 ± 0.45	11.21 ± 1.01	10.81 ± 0.95	9.83 ± 1.09	11.20 ± 0.58	11.64 ± 0.74
20:1n-9	0.65 ± 0.27	1.66 ± 0.06	0.86 ± 0.44	0.57 ± 0.23	0.53 ± 0.18	0.64 ± 0.39
24:1n-9	4.15 ± 0.77	2.05 ± 0.42	2.57 ± 0.73	3.36 ± 0.41	3.75 ± 1.02	2.99 ± 0.84
18:2n-6	0.58 ± 0.06	1.78 ± 0.32	0.43 ± 0.046	0.85 ± 0.15	0.64 ± 0.04	0.70 ± 0.13
18:3n-3	0.58 ± 0.12	0.76 ± 0.12	0.64 ± 0.22	0.50 ± 0.09	0.33 ± 0.25	0.49 ± 0.06
18:4n-3	0.00	0.00	0.00	0.06 ± 0.14	0.11 ± 0.24	0.00
20:4n-6	5.63 ± 1.66	2.26 ± 0.62	1.27 ± 0.32	4.83 ± 0.30	4.67 ± 0.49	4.27 ± 0.71
20:4n-3	0.28 ± 0.04	0.82 ± 0.14	0.55 ± 0.21	0.51 ± 0.20	0.43 ± 0.31	0.66 ± 0.49
22:4n-6	0.76 ± 0.20	0.74 ± 0.25	0.24 ± 0.07	0.30 ± 0.07	0.68 ± 0.94	0.44 ± 0.18
20:5n-3	4.60 ± 1.16	4.38 ± 0.45	4.86 ± 0.37	6.30 ± 0.79	5.54 ± 0.34	4.77 ± 0.97
22:5n-3	1.22 ± 0.13	2.24 ± 0.96	1.09 ± 0.13	1.20 ± 0.31	0.95 ± 0.30	0.98 ± 0.26
22:6n-3	41.30 ± 3.12	28.70 ± 2.71	39.24 ± 5.15	34.21 ± 2.65	39.81 ± 2.53	41.63 ± 3.19

[a]Data are given in mol% as means and standard deviations for five different samples.

TABLE 6
Fatty Acid Composition[a] of Phosphatidylethanolamine from White Muscle of Six Tuna Species

Acid	Tuna species					
	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
14:0	3.05 ± 1.07	2.94 ± 0.34	4.12 ± 0.39	2.82 ± 0.73	2.25 ± 1.01	3.32 ± 0.68
15:0	0.66 ± 0.14	0.91 ± 0.11	1.17 ± 0.08	1.06 ± 0.55	0.74 ± 0.22	0.93 ± 0.50
16:0	11.60 ± 1.25	11.97 ± 1.39	16.38 ± 0.25	12.80 ± 2.38	11.83 ± 1.74	12.27 ± 1.86
17:0	1.67 ± 0.37	1.54 ± 0.28	1.95 ± 0.50	1.63 ± 0.49	1.71 ± 0.19	1.54 ± 0.35
18:0	19.67 ± 3.24	21.82 ± 1.40	18.36 ± 1.00	22.84 ± 3.50	23.25 ± 1.52	17.85 ± 1.28
20:0	0.72 ± 0.25	0.83 ± 0.10	1.07 ± 0.08	1.12 ± 0.31	0.00	1.40 ± 0.61
24:0	0.86 ± 0.13	1.48 ± 0.91	2.47 ± 0.851	1.34 ± 0.38	1.05 ± 0.61	0.98 ± 0.61
16:1n-5	0.65 ± 0.43	0.91 ± 0.52	2.41 ± 0.23	0.89 ± 0.50	0.83 ± 0.12	1.28 ± 0.20
16:1n-7	1.07 ± 0.19	1.16 ± 0.32	2.19 ± 0.09	1.42 ± 0.38	1.42 ± 0.50	2.28 ± 1.26
16:1n-9	0.76 ± 0.49	0.77 ± 0.64	1.23 ± 0.61	0.66 ± 0.49	0.39 ± 0.27	1.13 ± 1.06
18:1n-7	1.26 ± 0.33	1.28 ± 0.19	2.18 ± 0.26	1.23 ± 0.87	1.36 ± 0.81	1.35 ± 0.14
18:1n-9	7.07 ± 1.03	8.05 ± 0.73	9.89 ± 0.39	7.60 ± 2.38	7.66 ± 1.64	11.24 ± 1.18
20:1n-9	1.57 ± 0.34	1.84 ± 0.41	3.58 ± 0.52	1.48 ± 0.91	1.10 ± 0.66	1.44 ± 0.89
24:1n-9	4.73 ± 1.40	3.09 ± 0.89	2.54 ± 0.78	4.14 ± 1.18	3.87 ± 0.37	4.17 ± 1.57
18:2n-6	1.07 ± 0.43	1.50 ± 0.25	2.13 ± 0.10	1.09 ± 0.27	1.44 ± 1.06	1.60 ± 0.32
18:3n-3	0.58 ± 0.12	0.76 ± 0.12	0.64 ± 0.22	0.50 ± 0.09	0.33 ± 0.25	0.49 ± 0.06
18:4n-3	0.00	0.00	0.00	0.00	0.14 ± 0.32	0.00
20:4n-6	3.98 ± 0.57	4.33 ± 0.63	2.48 ± 0.097	4.77 ± 1.62	4.75 ± 1.17	3.61 ± 0.83
20:4n-3	0.98 ± 0.29	0.68 ± 0.48	0.99 ± 0.309	0.45 ± 0.32	1.02 ± 0.36	1.08 ± 1.01
22:4n-6	2.93 ± 4.72	1.27 ± 0.83	1.57 ± 0.739	1.44 ± 2.31	0.48 ± 0.20	1.25 ± 0.64
20:5n-3	2.65 ± 0.99	3.64 ± 1.00	4.39 ± 0.100	2.45 ± 0.78	3.88 ± 1.70	2.62 ± 0.63
22:5n-3	1.33 ± 0.28	1.88 ± 0.40	1.93 ± 0.11	2.00 ± 0.74	0.99 ± 0.22	1.01 ± 0.70
22:6n-3	30.89 ± 1.87	26.84 ± 4.53	21.61 ± 2.46	25.42 ± 3.81	28.91 ± 6.14	25.60 ± 3.95

[a]Data are given in mol% as means and standard deviations for five different samples.

the same diglyceride precursor (54). On the other hand, this finding is supported by the alkenyl ether composition data mentioned previously (abundance of 18:0 in PE–PLA and of 16:0 in PC–PLA).

The preference of 16:0 over 18:0 as a saturated acid is repeated among the PL classes with choline as the polar head group, such as SPH and LPC. On the contrary, PI shows the inverse situation with a great abundance of 18:0.

TABLE 7
Fatty Acid Composition[a] of Lysophosphatidylcholine from White Muscle of Six Tuna Species

Acid	Tuna species					
	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
14:0	1.74 ± 0.49	3.40 ± 1.40	2.63 ± 0.87	3.86 ± 0.61	1.95 ± 0.65	3.12 ± 0.68
15:0	0.52 ± 0.06	0.93 ± 0.31	1.72 ± 0.67	1.35 ± 0.28	0.64 ± 0.34	1.06 ± 0.43
16:0	9.08 ± 2.16	13.99 ± 4.45	11.40 ± 0.96	20.52 ± 3.38	7.68 ± 1.78	9.99 ± 3.64
17:0	0.66 ± 0.14	1.07 ± 0.44	0.00	1.63 ± 0.25	0.72 ± 0.13	1.04 ± 0.33
18:0	3.21 ± 0.78	5.40 ± 2.29	7.51 ± 1.28	7.74 ± 1.06	2.38 ± 0.62	4.11 ± 1.50
20:0	0.65 ± 0.07	1.44 ± 0.96	0.00	1.764 ± 0.19	0.00	1.30 ± 0.54
24:0	0.58 ± 0.18	1.16 ± 0.27	0.00 ± 0.00	1.51 ± 0.31	0.62 ± 0.30	1.62 ± 0.72
16:1n-5	0.66 ± 0.14	1.23 ± 0.69	0.93 ± 0.25	2.10 ± 0.38	0.78 ± 0.21	0.87 ± 0.27
16:1n-7	0.96 ± 0.04	3.27 ± 1.06	3.72 ± 0.74	2.51 ± 0.51	1.55 ± 1.26	2.99 ± 1.37
16:1n-9	0.40 ± 0.29	0.69 ± 0.29	0.36 ± 0.14	0.74 ± 0.42	0.36 ± 0.13	0.47 ± 0.17
18:1n-7	1.33 ± 0.18	0.93 ± 0.32	1.69 ± 0.07	1.75 ± 0.32	1.04 ± 0.19	1.35 ± 0.11
18:1n-9	12.33 ± 1.52	14.46 ± 3.51	11.67 ± 0.17	13.80 ± 1.13	15.91 ± 1.97	13.35 ± 2.30
20:1n-9	1.02 ± 0.33	1.25 ± 1.91	2.16 ± 0.40	2.01 ± 1.11	0.52 ± 0.46	0.87 ± 0.43
24:1n-9	2.28 ± 1.58	1.11 ± 1.15	1.36 ± 0.17	1.81 ± 0.98	0.57 ± 0.300	1.20 ± 0.38
18:2n-6	0.95 ± 0.17	1.30 ± 0.78	2.27 ± 0.31	1.36 ± 0.57	1.02 ± 0.36	1.51 ± 0.42
20:4n-3	0.56 ± 0.43	0.49 ± 0.27	0.29 ± 0.06	1.15 ± 0.52	0.68 ± 0.45	0.57 ± 0.36
20:4n-6	6.19 ± 0.46	5.19 ± 1.31	2.40 ± 0.18	4.27 ± 1.30	7.38 ± 1.22	4.44 ± 0.89
22:4n-6	4.89 ± 0.32	3.65 ± 1.07	3.69 ± 0.88	4.26 ± 0.5	4.83 ± 0.37	4.33 ± 0.84
20:5n-3	4.81 ± 0.59	5.54 ± 2.17	5.59 ± 0.20	3.86 ± 0.30	6.32 ± 2.13	4.65 ± 0.77
22:5n-3	1.21 ± 0.14	1.18 ± 0.37	3.71 ± 1.33	1.58 ± 0.41	1.36 ± 0.16	1.48 ± 0.46
22:6n-3	45.26 ± 2.03	35.70 ± 6.33	34.71 ± 2.12	17.98 ± 3.85	42.90 ± 4.03	37.73 ± 6.83

[a]Data are given in mol% as means and standard deviations for five different samples.

TABLE 8
Fatty Acid Composition[a] of Phosphatidylinositol from White Muscle of Six Tuna Species

Acid	Tuna species					
	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
14:0	3.37 ± 1.1	4.57 ± 1.41	3.16 ± 0.55	3.86 ± 1.55	2.83 ± 0.71	2.77 ± 0.04
15:0	0.77 ± 0.18	1.62 ± 0.46	1.09 ± 0.09	1.28 ± 0.43	0.93 ± 0.16	1.27 ± 0.55
16:0	12.98 ± 1.94	15.52 ± 3.14	12.29 ± 1.42	13.91 ± 0.77	11.29 ± 1.38	12.66 ± 1.41
17:0	1.96 ± 0.86	2.15 ± 0.48	2.13 ± 0.24	2.05 ± 0.06	1.99 ± 0.19	2.02 ± 0.12
18:0	21.14 ± 4.64	14.70 ± 4.12	16.54 ± 0.56	24.02 ± 2.31	25.60 ± 1.73	24.42 ± 2.98
20:0	1.45 ± 1.04	1.12 ± 0.34	1.58 ± 0.72	0.47 ± 0.48	0.00 ± 0.00	0.75 ± 0.53
24:0	0.96 ± 0.00	1.73 ± 0.86	1.66 ± 0.72	1.28 ± 0.78	0.85 ± 0.87	1.55 ± 0.72
16:1n-5	0.70 ± 0.30	1.49 ± 0.14	0.56 ± 0.01	1.00 ± 0.38	0.78 ± 0.38	1.09 ± 0.52
16:1n-7	1.84 ± 0.35	2.96 ± 1.54	2.57 ± 0.20	2.15 ± 0.50	2.21 ± 1.50	2.75 ± 0.57
16:1n-9	0.54 ± 0.43	1.18 ± 0.76	1.62 ± 0.42	0.48 ± 0.15	0.98 ± 0.53	0.46 ± 0.18
18:1n-7	1.27 ± 0.42	1.89 ± 0.62	2.37 ± 0.15	1.44 ± 0.31	1.20 ± 0.27	1.20 ± 0.08
18:1n-9	8.32 ± 1.08	12.04 ± 3.35	17.78 ± 0.82	7.66 ± 1.15	8.13 ± 0.05	8.77 ± 1.82
20:1n-9	1.93 ± 1.12	2.76 ± 1.18	3.25 ± 0.79	0.52 ± 0.52	0.79 ± 0.24	1.23 ± 0.29
24:1n-9	4.34 ± 2.54	4.06 ± 2.03	2.72 ± 0.31	4.19 ± 1.55	3.56 ± 1.86	4.31 ± 1.11
18:2n-6	0.91 ± 0.06	1.04 ± 0.06	2.09 ± 0.05	1.39 ± 0.28	1.01 ± 0.03	1.25 ± 0.19
18:3n-3	0.64 ± 0.28	1.15 ± 0.69	1.21 ± 0.40	0.81 ± 0.12	0.47 ± 0.05	1.55 ± 0.77
20:4n-6	5.09 ± 2.35	5.17 ± 1.18	1.51 ± 0.07	3.60 ± 0.37	5.53 ± 1.20	4.64 ± 0.61
20:4n-3	0.43 ± 0.11	0.37 ± 0.16	0.43 ± 0.01	0.68 ± 0.25	0.38 ± 0.66	1.01 ± 0.57
22:4n-6	6.02 ± 2.43	1.55 ± 0.63	0.64 ± 0.08	0.79 ± 0.79	3.89 ± 2.71	1.49 ± 0.65
20:5n-3	2.18 ± 1.29	4.34 ± 3.04	4.85 ± 1.17	2.12 ± 0.66	3.68 ± 1.95	2.11 ± 0.89
22:5n-3	1.00 ± 0.59	0.79 ± 0.23	1.34 ± 0.16	1.23 ± 1.06	0.80 ± 0.01	1.69 ± 0.84
22:6n-3	24.05 ± 3.63	16.75 ± 5.69	19.18 ± 1.49	2.722 ± 2.63	25.32 ± 2.43	23.76 ± 1.62

[a]Data are given in mol% as means and standard deviations for five different samples.

The major monoene, 18:1n-9, was found in similar proportions in all PL classes, although choline-type PLs presented higher levels. Other monoenes, like 20:1n-9 and a homologue in the elongation pathway, 24:1n-9, were found to be highly concentrated in PE, PI, and PS. This distribution was in agreement with previous results reported in the case of the white muscles of albacore tuna and cod (39,46).

With respect to PUFA, 20:5n-3 and 22:6n-3 were especially concentrated in PC and LPC with levels around 5–6 and 30–45% of each fatty acid, respectively. Relatively high con-

TABLE 9
Fatty Acid Composition[a] of Phosphatidylserine from White Muscle of Six Tuna Species

Acid	Tuna species					
	Bigeye	Bluefin	Bonito	Frigate	Skipjack	Yellowfin
14:0	3.80 ± 1.20	4.90 ± 1.26	4.59 ± 1.12	5.31 ± 1.72	5.37 ± 1.60	4.67 ± 2.00
15:0	1.21 ± 0.24	1.37 ± 0.22	1.76 ± 0.47	1.64 ± 0.18	1.48 ± 0.30	1.81 ± 0.29
16:0	16.70 ± 3.12	16.61 ± 1.08	19.29 ± 1.63	17.98 ± 1.72	15.37 ± 1.28	17.52 ± 1.40
17:0	1.89 ± 0.67	1.73 ± 0.44	3.42 ± 1.29	1.88 ± 0.42	1.88 ± 0.36	2.00 ± 0.52
18:0	16.79 ± 2.48	15.39 ± 2.48	12.77 ± 0.60	17.46 ± 3.09	18.84 ± 3.10	16.81 ± 2.57
20:0	2.25 ± 1.02	1.13 ± 0.28	1.34 ± 0.69	1.69 ± 0.56	0.00 ± 0.00	1.16 ± 0.70
24:0	1.01 ± 0.68	2.33 ± 1.89	1.41 ± 0.324	2.20 ± 1.08	2.45 ± 1.15	1.88 ± 1.13
16:1n-5	3.33 ± 3.78	1.41 ± 1.81	1.82 ± 0.28	1.64 ± 0.65	1.59 ± 0.59	1.88 ± 0.60
16:1n-7	2.32 ± 0.68	2.18 ± 0.63	2.47 ± 0.09	3.16 ± 0.85	3.52 ± 0.61	4.03 ± 0.19
16:1n-9	1.51 ± 0.93	1.17 ± 0.59	0.56 ± 0.08	0.70 ± 0.09	0.88 ± 0.34	0.76 ± 0.21
18:1n-7	2.00 ± 0.34	1.88 ± 0.72	1.67 ± 0.08	1.43 ± 0.56	1.27 ± 0.47	1.54 ± 0.20
18:1n-9	10.93 ± 1.57	12.65 ± 1.23	13.77 ± 1.31	11.42 ± 1.40	12.36 ± 2.48	14.46 ± 2.74
20:1n-9	1.81 ± 0.55	1.79 ± 0.87	4.38 ± 1.46	1.99 ± 0.65	1.59 ± 0.82	2.73 ± 1.28
24:1n-9	5.02 ± 1.52	3.44 ± 1.00	2.65 ± 0.73	3.47 ± 2.49	3.54 ± 0.63	3.56 ± 2.18
18:2n-6	1.34 ± 0.23	1.48 ± 0.53	2.69 ± 0.45	2.22 ± 0.95	1.65 ± 0.79	2.21 ± 0.84
18:3n-3	1.48 ± 1.10	2.12 ± 0.41	0.94 ± 0.19	1.51 ± 0.59	1.56 ± 0.81	1.30 ± 0.25
20:4n-6	3.25 ± 1.20	2.95 ± 2.28	2.02 ± 0.41	3.19 ± 1.05	2.53 ± 1.72	2.32 ± 1.41
20:4n-3	0.15 ± 0.34	0.37 ± 0.44	0.91 ± 0.08	0.49 ± 0.45	0.000 ± 0.00	0.12 ± 0.26
22:4n-6	1.59 ± 1.64	1.77 ± 1.12	2.37 ± 0.38	1.70 ± 0.87	1.51 ± 0.38	1.46 ± 0.58
20:5n-3	2.97 ± 2.36	2.89 ± 1.96	3.12 ± 1.29	2.71 ± 1.12	3.29 ± 1.86	2.79 ± 1.47
22:5n-3	1.34 ± 0.43	2.25 ± 1.36	1.52 ± 0.18	1.53 ± 0.92	2.37 ± 1.38	2.24 ± 1.88
22:6n-3	17.44 ± 5.71	14.62 ± 2.30	15.26 ± 2.80	14.11 ± 1.74	16.76 ± 5.70	12.47 ± 3.53

[a]Data are given in mol% as means and standard deviations for five different samples.

tents also were found in PE. Results obtained herein do not confirm the idea that PE should concentrate a higher PUFA content than any other PL class (18,38). PI and PS showed a more saturated composition with relatively low values of 22:6n-3 fatty acid (around 25 and 15%, respectively).

The high PUFA content found in LPC may be related to the enzymatic hydrolysis of PC occurring during storage at low temperatures. Some authors have found that this lipolysis takes place first with saturated fatty acids that are mainly esterified in *sn*-1 position of the PL moiety, and in a second step with PUFA (14,55,56). As a result, a high concentration in PUFA could be obtained in the remaining lysophospholipids. The findings obtained in this research were in accordance with that notion.

However, a different composition was found in LPC of frigate as compared to the other species—saturated fatty acids (16:0 and 18:0) showed high contents, while n-3 PUFA (20:5 and 22:6) were present in lower contents. The proportion of these acids in PC was not significantly different from other species; hence, reasons related to a partial hydrolysis of PC could not explain the low content found in LPC. More complex factors surely may be considered, such as diet, temperature, salinity, or even internal conditions (18).

From a nutritional point of view, the enzymatic hydrolysis occurring in all samples did not seriously affect the n-3-composition, as can be inferred from the comparison of fatty acid composition of PC and PE in samples which showed higher levels of LPC, such as skipjack and yellowfin in contrast with bonito or frigate.

Finally, special emphasis often is given to the n-3/n-6 PUFA ratio for marine and freshwater fish actually consumed by humans (37,57). As could be expected, all PL classes described in this work were characterized by high ratios of n-3 to n-6 polyenes. Special attention must be given to the fatty acid composition of PI. Recently, several authors (52,58,59) have reported that PI from some marine fish tissues is especially enriched in 20:4n-6. In our case, PI showed a lower n-3/n-6 ratio than other PL classes, but arachidonic acid was not the major PUFA in any tuna species. This situation already has been described in white muscle of cod (39), in contrast with previous reports.

A different situation could be observed in the relative contents of 20:5n-3 and 20:4n-6. In general, PE and PI showed a larger proportion of 20:4n-6 than that of 20:5n-3, and PC revealed the opposite (Tables 5, 6, and 8). Studies carried out on fish coming from Australian waters contained different relative contents of both acids, depending on the species (60,61). In addition, an important variation in the content of 20:4n-6 found in PC and LPC also was observed between the six tuna species (Tables 5 and 7). In these classes, bonito showed the highest values of the n-3/n-6 ratio.

The present research indicates that the PL of white muscle of fresh tuna species has a noteworthy nutritional value, supplying substantial amounts of PUFA and PLA. No significant differences were found in the distribution of the PL classes among the different tuna species studied. Furthermore, in terms of fatty acid and alkenyl ether compositions, PL classes showed some general similarities, although ratios between certain fatty acids were found to be different.

ACKNOWLEDGMENTS

We acknowledge financial support for Research Project ALI 90-0773 provided by CICYT and Research Project CEE UP. 2.571 (of DG XIV).

REFERENCES

1. Alimarket, Informe anual de alimentación 92 (1992), Publicaciones Profesionales S.A., Madrid.
2. Pigott, G., and Tucker, B. (1987) Science Opens New Horizons for Marine Lipids in Human Nutrition, *Food Rev. Intern. 3*, 105–138.
3. Ackman, R. (1989) *Marine Biogenic Lipids, Fats and Oils*, Vol. 1, pp. 103–107, CRC Press, Inc., Boca Raton.
4. Chan, H. (1987) *Autoxidation of Unsaturated Lipids*, Academic Press, New York, pp. 17–50.
5. Hsieh, R., and Kinsella, J. (1989) Oxidation of Polyunsaturated Fatty Acids: Mechanisms, Products and Inhibition with Enphasis on Fish, *Adv. Food Res. and Nutr. Res. 33*, 233–341.
6. Caroll, K., and Braden, L. (1986) Differing Effects of Dietary Polyunsaturated Vegetable and Fish Oils of Mammary Tumorigenesis, *Prog. Lipid Res. 25*, 583–585.
7. Lees, R., and Karel, M. (1990) *Omega-3 Fatty Acids in Health and Disease*, Marcel Dekker Inc., New York and Basel, Chapter 2.
8. Gallardo, J., Aubourg, S., and Pérez-Martín, R. (1989) Lipid Classes and Their Fatty Acids at Different Loci of Albacore (*Thunnus alalunga*): Effects of Precooking, *J. Agric. Food Chem. 37*, 1060–1064.
9. Ohshima, T., and Koizumi, C. (1983) Accumulation of Lysophosphatidylcholine and Lysophosphatidylethanolamine in Muscle of Fresh Skipjack, *Bull. Jap. Soc. Scient. Fisher. 49*, 1205–1212.
10. Aubourg, S., Pérez-Martín, R., and Gallardo, J. (1989) Stability of Lipids of Frozen Albacore (*Thunnus alalunga*) During Steam Cooking, *Int. J. Food Sci. Tech. 24*, 341-345.
11. Melva, P., Tsukuda, N., and Okada, M. (1982) Content and Composition of Lipids in Peruvian Canned Fish, *Bull. Tokai Reg. Fish. Res. Lab. 106*, 89–96.
12. Balogun, A., and Talabi, S. (1984) An Investigation into Lipid Classes of Skipjack Tuna (*Katsuwonus pelamis*), *J. Food Sci. 49*, 1638–1639.
13. Aubourg, S., Sotelo, C., and Gallardo, J. (1990) Zonal Distribution of Fatty Acids in Albacore *(Thunnus alalunga)* Triglycerides and Their Changes During Cooking, *J. Agric. Food Chem. 38*, 809–812.
14. Ohshima, T., Wada, S., and Koizumi, C. (1984) Preferential Enzymatic Hydrolysis of Phosphatidylcholine in Skipjack Flesh During Frozen Storage, *Bull. Jap. Soc. Scient. Fisher. 50*, 2091–2098.
15. Ohshima, T., Wada, S., and Koizumi, C. (1989) 1-*O*-Alk-1′-Enyl-2-Acyl and 1-*O*-Alkyl-2-Acyl Glycerophospholipids in White Muscle of Bonito *Euthynnus pelamis* (Linnaeus), *Lipids 24*, 363–370.
16. Takahashi, K., Ebina, H., Egi, M., Matsumoto, K., and Zama, K. (1985) *Bull. Jap. Soc. Scient. Fisher. 51*, 1475–1486.
17. King, M., Boyd, L., and Sheldon, B. (1992) Antioxidant Properties of Individual Phospholipids in a Salmon Oil Model System, *69*, 545–551.
18. Pearson, A., Love, J., and Shorland, F. (1977) Warmed over Flavor in Meat, Poultry and Fish, *Adv. Food Res. 23*, 2–74.
19. Litchfield, C. (1972) *Analysis of Triglycerides*, Academic Press, New York, Chapter 12.
20. Litchfield, C. (1972) Taxonomic Patterns in the Triglyceride Structure of Natural Fats, *Fette, Seifen, Anstrichm. 74*, 223–230.
21. Rugraff, L., and Karleskind, A. (1983) Analyse des melanges de graisses animales. Application au contrôle de l'absence de graisse de porc dans les suifs et subsidiairement dans les produits carnés et dérivés crus ou cuits, *Rev. Franç. Corps Gras 30-9*, 323–331.
22. Bligh, L., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification., *Biochem. Physiol. 37*, 911–917.
23. Herbes, S.E., and Allen, C.P. (1983) Lipid Quantification of Freshwater Invertebrates: Method Modification for Microquantitation, *Can. J. Fish Aquat Sci. 40*, 1315–1317.
24. Lowry, R., and Tinsley, I. (1976) Rapid Colorimetric Determination of Free Fatty Acids, *J. Am. Oil Chem. Soc. 53*, 470–472.
25. Raheja, R., Kaur, C., Singh, A., and Bhatia, Y. (1973) New Colorimetric Method for the Quantitative Determination of Phospholipids Without Acid Digestion, *J. Lipid Res. 14*, 695–697.
26. Bradová, V., Smid, F., Ledvinová, J., and Michalec, C. (1990) Improved One-Dimensional TLC for the Separation of Phospholipids in Biological Material, *J. Chromatogr. 533*, 297–303.
27. Bitman, J., and Wood, D.L. (1982) An Improved Copper Reagent for Quantitative Densitometric Thin-Layer Chromatography of Lipids, *J. Chromatogr. 5–6*, 1155–1162.
28. Lepage, G., and Roy, C. (1986) Direct Transesterification of Cell Classes of Lipids in a One Step Reaction, *J. Lipid Res. 27*, 114–120.
29. Visnaanathan C., Basilio, M., Hoeret, S., and Lundberg, W. (1978) Reaction Thin-Layer Chromatography in the Analysis of Mixtures of Alkenyl Acyl- and Diacylphosphatides, *J. Chromatogr. 34*, 241–245.
30. Stansby, E. (1973) Polyunsaturates and Fat in Fish Flesh, *J. Amer. Diet. Assoc. 63*, 625–630.
31. Ackman, R. (1979) in *Advances in Fish Science and Technology* (Connell, J.J., ed.) Fishing News Books Ltd., Farnham, Surrey, England, pp. 86–103.
32. Dotson, R.C. (1976) in *The Physiological Ecology of Tunas* (Sharp, D., and Dizon, A.E, eds.) Academic Press, New York, Chapter 6.
33. Hardy, R., and Smith, J.G.M. (1976) The Storage of Mackerel (*Scomber scombrus*). Development of Histamine and Rancidity, *J. Sci. Food Agric. 27*, 595–601.
34. Hardy, R., McGill, A.S., and Gunstone, F.D. (1979) Lipid and Autoxidative Changes in Cold Stored *(Gadus morthua)*, *J. Sci. Food Agric. 30*, 999–1006.
35. Stansby, M.E. (1961) in *Proximate Composition of Fish, Fish in Nutrition*, International Congress, Washington D.C.
36. Shuster, C.Y., Froines, J.R., and Olcott, H.S. (1963) Phospholipids of Tuna White Muscle, *J. Am. Oil Chem. Soc. 41*, 36–41.
37. Henderson, J.R., and Tocher, D.R. (1987) The Lipid Composition and Biochemistry of Freshwater Fish, *Prog. Lipid. Res. 26*, 281–347.
38. Vaskowski, V. (1989) Phospholipids, in *Marine Biogenic Lipids, Fats and Oils* (Ackman, R., ed.) Vol.1, pp. 435–456, CRC Press, Inc., Boca Raton.
39. Lie, O., and Lambertsen, G. (1991) Fatty Acid Composition in Seven Tissues of Cod (*Gadus morhua*), Determined by Combined HPLC and Gas Chromatography, *J. Chromatogr. 565*, 119–129.
40. Parker, R.S., Selivonchick, D.P., and Sinnhuber, R.O. (1980) Turnover of Label [1-^{14}C]Linolenic Acid in Phospholipids of Coho Salmon, *Oncorhynchus kitsutch*, *Lipids 15*, 80–84.
41. Bleasdale, J.E., Hawthorne, J.N., Widlund, L., and Heilbronn, E. (1976) Phospholipid Turnover in *Torpedo marmorata* Electric Organ During Discharge *in vivo*, *Biochem. J. 158*, 557–565.
42. Ohshima, T., Wada, S., and Koizumi, C. (1985) Accumulation of Lyso-Form Phospholipids in Several Species of Fish Flesh During Storage at −5°C, *Bull. Jap. Soc. Scient. Fisher. 51*, 965–971.
43. Belsare, D.K., and Belsare, S.D. (1976) Liver Phospholipid Distribution in Hypophysectomised Catfish, *Clarias batrachus* and *Heteropneustes fossils*, *Endokrinologie 67*, 365–368.

44. Woodtke, E. (1981) Temperature Adaption of Biological Membranes on the Unsaturation of the Main Neutral and Charged Phospholipids in Mitochondrial Membranes of the Carp *(Cyprinus carpio Lineo), Biochim. Biophys. Acta 640*, 698–709.
45. Blank, L.B., Cress, E.A., Smoth, Z.L., and Snyder, F. (1992) Meats and Fish Consumed in the American Diet Contain Substantial Amounts of Ether-Linked Phospholipids, *J. Nutr. 122*, 1656–1661.
46. Medina, I., Aubourg, S. and Pérez-Martín, R. (1993) Damage of 1-*O*-Alk-1-Enyl Glycerophospholipids of Albacore Tuna (*Thunnus alalunga*) During Thermal Processing, *J. Agric. Food Chem. 41*, 2395–2399.
47. Matheson, D.F., Oei, R., and Roots, B.Y. (1980) Changes in the Fatty Acyl Composition of Phospholipids in the Endothelial Cells and Mitochondria of Rat Brain, *Physiol. Zool. 53*, 57–69.
48. Jeong, B., Oshima, T., and Koimuzi, C. (1991) Changes in Fatty Acid Chain Compositions of Ether and Ester Glycerophospholipids of Japanese Oyster *crassostrea gigas* During Frozen Storage, *Nippon Suisan Gakkaishi 57*, 561–570.
49. Ohshima, T., Wada, S., and Koizumi, C. (1989) Molecular Species of 1-*O*-Alk-1-Enyl-2-Acylglycerophospholipids of Bonito White Muscle, *Nippon Suisan Gakkaishi 55*, 885–890.
50. Roots, B., and Johnston, P. (1968) Plasmalogens of the Nervous System and Environmental Temperature, *Comp. Biochem. Physiol. 26*, 553–560.
51. Chapelle, S., Hakanson, J., Nevenzel, J., and Benson, A. (1987) Ether Glycerophospholipids of Gills of Two Pacific Crabs *Cancer antennarius* and *Portunus xantusi, Lipids 22*, 76–79.
52. Linares, F., and Henderson, R.J. (1991) Incorporation of ^{14}C-Labelled Polyunsaturated Fatty Acids by Juvenile Turbot, *Scophtalmus maximus* (L.) *in vivo, J. Fish Biol. 38*, 335–347.
53. Nelson, G.J. (1962) Lipid Comparation of Normal Mouse Liver, *J. Lipid Res. 3*, 71–79.
54. Kennedy, E.P. (1957) Metabolism of Lipids, *Ann. Rev. Biochem. 26*, 119–148.
55. Viswanathan Nair, P., and Gopakumar, K. (1985) Selective Release of Fatty Acids During Lipid Hydrolysis in Frozen-Stored Milk Fish (*Chanos chanos*), *Fish. Technol. 22*, 1–4.
56. Medina, I., and Sacchi, R. (1994) Acyl Stereospecific Analysis of Tuna Phospholipids *via* High Resolution ^{13}C-NMR Spectroscopy, *Chem. Phys. Lipids 70*, 53–61.
57. Watanabe, T. (1982) Lipid Nutrition in Fish, *Comp. Biochem. Physiol. 73B*, 3–15.
58. Bell, H.V., Henderson, J., and Sargent, R. (1985) Molecular Species Analysis of Phosphoglycerides from the Ripe Roes of Cod (*Gadus morthua*), *Comp. Biochem. Physiol. 81B*, 193–198.
59. Tocher, D.R., and Sargent, J.R. (1984) Analysis of Lipids and Fatty Acids in Ripe Roes of Some Northwest European Marine Fish, *Lipids 19*, 492–499.
60. Gibson, R.A. (1983) Australian Fish–Excellent Source of both Arachidonic Acid and ω3 Polyunsaturated Fatty Acids, *Lipids 18*, 743–750.
61. Brown, A.J., Roberts, D.C.K., Pritchard, J.E., and Truswell, A.S. (1990) A Mixed Australian Fish Diet and Fish-Oil Supplementation-Impact on the Plasma Lipid Profile of Healthy Men, *Am. J. Clin. Nutr. 52*, 825–833.

[Received January 5, 1995, and in final revised form September 28, 1995; Revision accepted October 10, 1995]

Symposium on Palm Oil and Its Components

The Palm Oil Research Institute of Malaysia (PORIM) has pursued a policy of supporting biological research involving the use of palm oil, its fractions (palm olein, palm stearin, etc.), or other components such as Vitamin E or tocotrienols. At the occasion of the International Palm Oil Congress held in Kuala Lumpur, Malaysia, in September 1993, PORIM invited its grantees to present their findings. The papers which follow represent the completed projects of some of the PORIM grantees. I would like to thank Dr. David Kritchevsky for facilitating the submission of these manuscripts and for helping in the review process, and Dr. Wolfgang Baumann, former Editor-in-Chief of *Lipids*, for his meticulous attention to assuring their optimal presentation.

Howard R. Knapp, M.D., Ph.D.
Editor-in-Chief, *Lipids*

Copyright © 1995 by AOCS Press

Effect of Tocotrienols on the Growth of a Human Breast Cancer Cell Line in Culture[1]

Kalanithi Nesaretnam[a,*] Najla Guthrie[b], Ann F. Chambers[c], and Kenneth K. Carroll[b]

[a]Palm Oil Research Institute of Malaysia, Kuala Lumpur 50720, Malaysia, [b]University of Western Ontario, London, Ontario N6A 5Cl and [c]London Regional Cancer Research Centre, London, Ontario, N6A 4L6 Canada

ABSTRACT: The tocotrienol-rich fraction (TRF) of palm oil consists of tocotrienols and some α-tocopherol (α-T). Tocotrienols are a form of vitamin E having an unsaturated side-chain, rather than the saturated side-chain of the more common tocopherols. Because palm oil has been shown not to promote chemically-induced mammary carcinogenesis, we tested effects of TRF and α-T on the proliferation, growth, and plating efficiency (PE) of MDA-MB-435 estrogen-receptor-negative human breast cancer cells. TRF inhibited the proliferation of these cells with a concentration required to inhibit cell proliferation by 50% of 180 μg/mL, whereas α-T had no effect at concentrations up to 1000 μg/mL as measured by incorporation of [^{3}H]thymidine. The effects of TRF and α-T also were tested in longer-term growth experiments, using concentrations of 180 and 500 μg/mL. We found that TRF inhibited the growth of these cells by 50%, whereas α-T did not. Their effect on the ability of these cells to form colonies also was studied, and it was found that TRF inhibited PE, whereas α-T had no effect. These results suggest that the inhibition is due to the presence of tocotrienols in TRF rather than α-T.

Lipids 30, 1139–1143 (1995).

Earlier studies have shown that palm oil does not promote chemically-induced mammary tumors in rats like other dietary fats and oils (1–3). Furthermore, the growth of cells derived from a spontaneously arising mouse mammary tumor, when injected into animals pre-fed with various dietary lipids, was halved when 20% (of energy) palm oil was fed compared with 20% safflower oil, and was reduced to one-sixth in animals fed 5% palm oil (4). It has been suggested that this inhibition might be due to the fact that palm oil is not rich in linoleic acid, which has been shown to promote cancer in *in vivo* and *in vitro* studies (5–8). A second explanation may be found in the anticarcinogenic potential of palm oil's minor components, mainly its vitamin E tocotrienols, as recent studies have shown that palm oil stripped of the vitamin E fraction enhances tumorigenesis. It also was demonstrated that addition of the tocotrienol-rich fraction (TRF) isolated from palm oil to corn oil caused a delay in the appearance of tumors when compared with the corn oil control group (9). Tocotrienols also caused a delay in the onset of subcutaneous lymphoma by 2–4 wk in HRS/J hairless mice, a strain genetically susceptible to subcutaneous lymphoma (10). The life span of mice inoculated with transplanted tumor cells was increased with tocotrienols (11). An inhibition of tumor cell growth of Hela and p388 cells by tocotrienols in culture also has been reported (12). The two forms of vitamin E—tocopherol and tocotrienol—were tested for chemopreventive activity in two chemically-induced rat mammary tumor models. It was found that only the tocotrienol group had a statistically significant increase in tumor latency when mammary tumors were induced with 7,12-dimethylbenz(a)antracene (DMBA), and neither analogue modified latency when nitrosometylurea was used (13).

Tocotrienols account for about 70% of the vitamin E present in palm oil and have an unsaturated side-chain, whereas tocopherols have a saturated side-chain. Although tocotrienols are generally believed to be much less biologically active than tocopherols, they have been shown to have greater free radical scavenging properties as cell membrane constituents (14,15). It has been proposed that vitamin E act as scavenger of nitrite compounds, thus preventing the formation of cancer-promoting nitrosamines (16). Epidemiological data suggest that vitamin E and other antioxidants may decrease cancer incidence. Several studies have revealed that the subjects with the highest serum concentrations of vitamin E have a lower risk of certain cancers (17–20). In addition to this role as a free radical scavenger, high intakes of vitamin E also may enhance the body's immune response (21).

Our interest in tocotrienols was stimulated by observations that dietary palm oil only promotes chemically-induced carcinogenesis in rats when stripped of the TRF. In this study, we tested the effect of TRF isolated from palm oil in comparison with α-tocopherol (α-T) on the proliferation and growth of MDA-MB-435 human breast cancer cells.

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, September 1993.

*To whom correspondence should be addressed at Palm Oil Research Institute of Malaysia, P.O. Box 10260, Kuala Lumpur 50720, Malaysia.

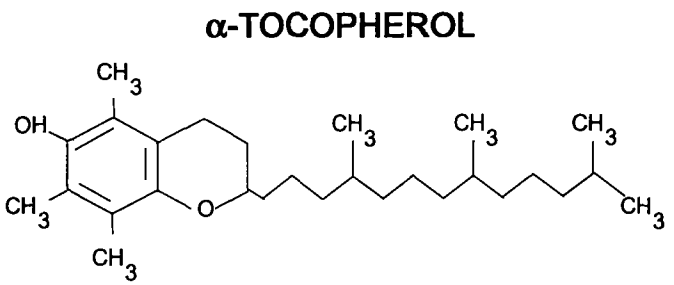
Abbreviations: α-T, α-tocopherol; DMBA, 7,12-dimethylbenz(a)anthracene; MTT, 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; PE, plating efficiency; TRF, tocotrienol-rich fraction.

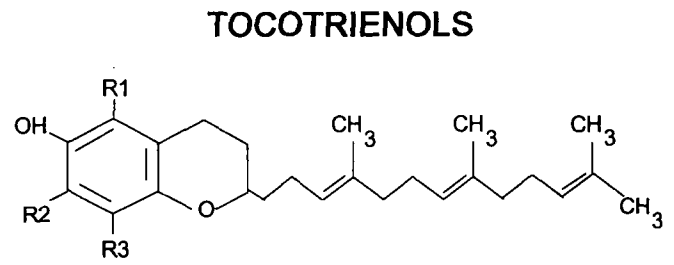
Copyright © 1995 by AOCS Press

MATERIALS AND METHODS

Materials. Tissue culture plastic ware, growth media, and serum for cell culturing were purchased from Gibco Laboratories (Burlington, Ontario, Canada). [^{3}H]Thymidine (6.7 Ci/mmol) was purchased from ICN (Irvine, CA). Scintiverse was purchased from Fisher Scientific (Nepean, Ontario, Canada). The TRF was obtained from the Palm Oil Research Institute of Malaysia (Kuala Lumpur, Malaysia). α-T was obtained from Sigma Chemical Co. (St. Louis, MO). The TRF and α-T were dissolved in dimethyl sulfoxide and diluted with culture medium. All other chemicals were purchased from Sigma Chemical Co.

Isolation of TRF. The extraction of TRF from palm oil is described by Sundram and Gapor (22) and was performed as follows: Palm fatty acid distillate was used as the starting material, and this was converted into methyl esters by esterification. The methyl esters were then removed by distillation, leaving behind a vitamin E concentrate. This was then concentrated by crystallization and passed through an ion exchange column, resulting in vitamin E that was 60–70% pure. Further purification of this preparation was achieved by washing and then drying the concentrate and passing it through a second molecular distillation stage. The final purity of the vitamin E preparation was 95–99% and its composition was: α-T, 32%; α-tocotrienol, 25%; γ-tocotrienol, 29%; and δ-tocotrienol, 14%. The structural formulae of the compounds are depicted in Figure 1.





	R1	R2	R3
α-tocotrienol	CH_3	CH_3	CH_3
γ-tocotrienol	H	CH_3	CH_3
δ-tocotrienol	H	H	CH_3

FIG. 1. Structures of α-tocopherol and tocotrienols.

Cell culture experiments. The human breast cancer cell line MDA-MB-435 was obtained from Dr. Janet Price (M.D. Anderson Hospital, Houston, TX) (23) and was cultured routinely in minimum essential medium supplemented with 10% fetal bovine serum in a 95% air/5% CO_2 incubator at 37°C. Stock cultures were seeded at a density of 2×10^5 and allowed to grow to 80–90% confluence in T-75 flasks. Culture media was changed every two to three days.

The proliferation assay was performed in a 96-well, flat-bottom plate at a plating density of 2×10^4 cells/well in a total volume of 200 μL of medium. The cells were incubated at 37°C for 48 h, with or without the test compounds. Fifty μL of the TRF was added at the following final concentrations: 1 mg/mL, 500 μg/mL, 250 μg/mL, 200 μg/mL, 180 μg/mL, 160 μg/mL, 140 μg/mL, 120 μg/mL, 60 μg/mL, and 30 μg/mL. Tocopherol controls at the same concentrations were carried out simultaneously. [^{3}H]Thymidine (0.5 μCi/well) was then added, and after 4 h the cells were trypsinized and harvested onto a glass fiber filter paper on a semi-automatic 12-well cell harvester (Skatron, Sterling, VA) for evaluation of thymidine incorporation into DNA. Radioactivity on the paper was counted using Scintiverse for 10 min in a liquid scintillation counter.

Plating efficiency (PE) was determined by seeding a known number of cells in 60- or 100-mm tissue culture dishes with or without a given concentration of either TRF or α-T and counting the colonies 14 d later. PE was calculated as the number of colonies divided by the number of cells seeded. Relative PE was calculated as experimental PE divided by control PE.

For growth experiments, the cells were plated at 1×10^4 cells/60 mm dish in 7 mL of medium. TRF was added at concentrations of 125 and 500 μg/mL. The same concentrations of α-T were used to make allowance for any effects due to tocopherols in the TRF. Dishes were incubated for 2, 4, 6, 8, and 10 d, after which the cells were trypsinized and counted with a hemocytometer.

Viability of cells was measured by the 3,(4,5-dimethylthiazole-2-yl)2,5diphenyltetrazolium bromide (MTT) assay as described by Hansen *et al.* (24). In this assay, MTT is converted to a blue formazan product by dehydrogenases that are active in living cells. The intensity of the blue color developed is a measure of cell viability. MDA-MB-435 cells (8×10^4/well) were seeded with various concentrations of TRF and α-T in a 96-well plate in a total volume of 200 μL of medium. MTT (25 μL of 5 mg/mL) was added to each well. After 3 h, 100 μL of extraction buffer consisting of 20% SDS dissolved in a 50% dimethyl formamide/50% water solution at pH 4.0 was added. The blue color formed was measured at 590 nm.

RESULTS

To investigate the effects of TRF and α-T on cell proliferation, we examined the incorporation of [^{3}H]thymidine into MDA-MB-435 cells in the presence of various concentrations of these substances after 48 h. At a concentration of 180 μg/mL,

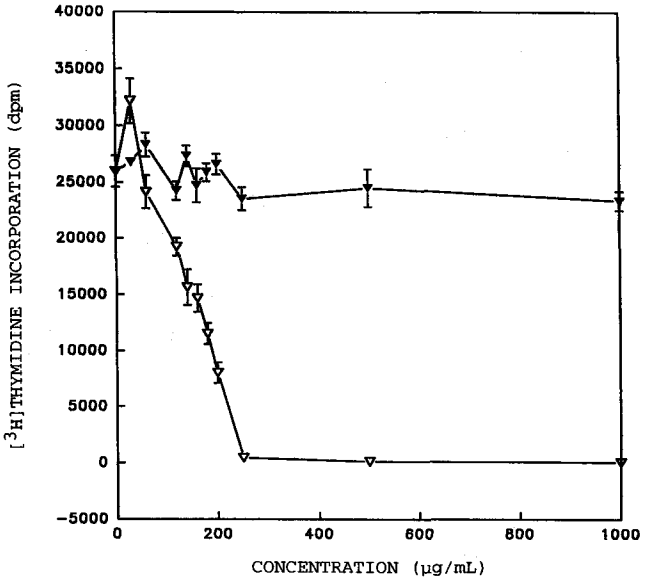


FIG. 2. Effects of α-tocopherol (α-T) (▼) and tocotrienol-rich fraction (TRF) (▽) on the proliferation of MDA-MB-435 cells. The cells were incubated with various concentrations of TRF or α-T (1 mg/mL or 30 µg/mL, respectively) for 48 h. [^{3}H]thymidine (0.5 µCi/well) was then added and the cells were harvested after 4 h to evaluate the incorporation of thymidine into DNA. Points are the average of mean values from three experiments ± SE.

TRF showed a 50% inhibitory effect (Fig. 2). At concentrations above 225 µg/mL, TRF completely inhibited the incorporation of [^{3}H]thymidine by MDA-MB-435 cells. However, there appeared to be a slight stimulation of thymidine incorporation by the cells at the lowest concentration (30 µg/mL) of TRF. In contrast, α-T did not show any inhibition of cell proliferation, even at the highest concentration of 1000 µg/mL.

Figure 3 shows the influence of TRF and α-T on the growth of the MDA-MB-435 cell line. Figure 3A shows the effect of TRF and α-T at a concentration 180 µg/mL on the cells. As shown earlier in the proliferation assay, TRF inhibited the growth of the cells to 50% of control values, and α-T did not. At a higher concentration of 500 µg/mL (Fig. 3B), TRF was shown to be extremely effective in producing complete growth suppression, whereas α-T had no effect.

The sensitivity of the human breast cancer cells to various concentrations of TRF or α-T was determined (Fig. 4). At 1000 µg/mL concentration of α-T, the PE of MDA-MB-435 cells was nearly identical to the control PE of the cells. At the same concentration of TRF, the PE of MDA-MB-435 cells was reduced by approximately three orders of magnitude.

The MTT assay uses the principle whereby cell growth or cell kill is indicated by the conversion or lack of conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically. As can be seen from Figure 5, the MDA-MB-435 cells incubated with various concentrations of α-T grew normally (no change in o.d. reading) and were comparable to the control cells, where there was no addition of α-T or TRF. The effect of various concentrations of TRF on the cells showed,

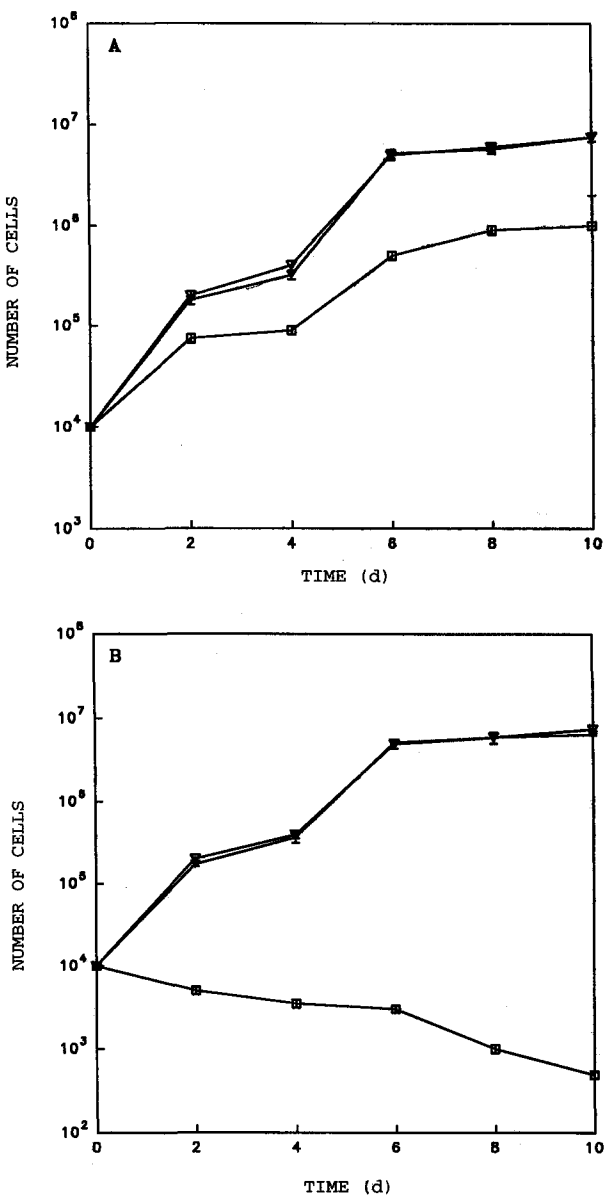


FIG. 3. Comparison of the inhibitory effects of α-tocopherol (α-T) (▼) and tocotrienol-rich fraction (TRF) (□) on MDA-MB-435 cell growth. TRF and α-T were added at concentrations of 180 (A) and 500 µg/mL (B). Neither TRF nor α-T was added to control cells (▽). Points are the average of mean values from three experiments ± SE.

however, that the cells were viable only up to 200 µg/mL; thereafter the o.d. curve dropped rapidly, indicating a substantial loss of cells.

DISCUSSION

As many dietary antioxidants have been shown to prevent cancer in animal studies, the potential activity of vitamin E (tocopherols and tocotrienols) is of interest. Although tocotrienols are known to possess a lower biological "vitamin E activity" than tocopherols (14,25), recent findings suggest that α-tocotrienol is a better antioxidant than α-T (15). In light of the fact that palm oil does not promote chemically-

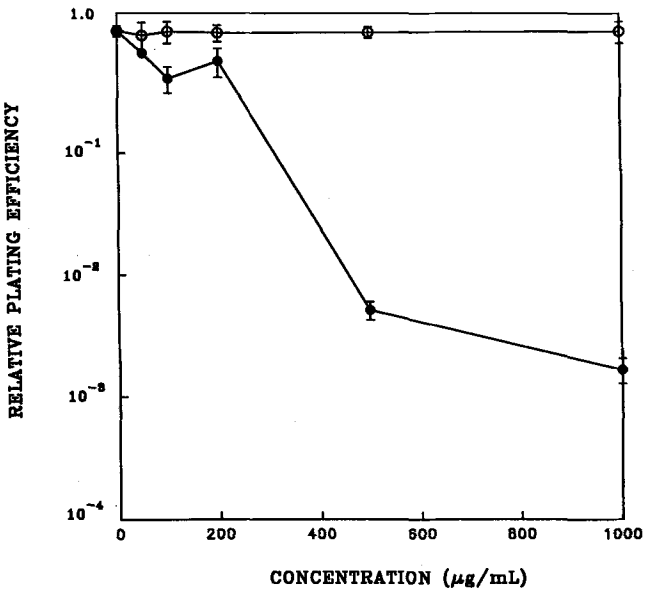


FIG. 4. Effect of tocotrienol-rich fraction (TRF) (●) and α-tocopherol (α-T) (○) on the plating efficiency (PE) of MDA-MB-435 cells relative to control PE without either compound. Cells were grown in 50- or 100-mm tissue culture dishes with or without a given concentration of either TRF or α-T. The colonies were counted 14 d later. Points are mean values ± SD.

induced mammary carcinogenesis when fed at a high level to rats in a semipurified diet, and this failure to promote appears to be due to the TRF of palm oil, we tested the effects of tocotrienols and α-T on human breast cancer cells in culture.

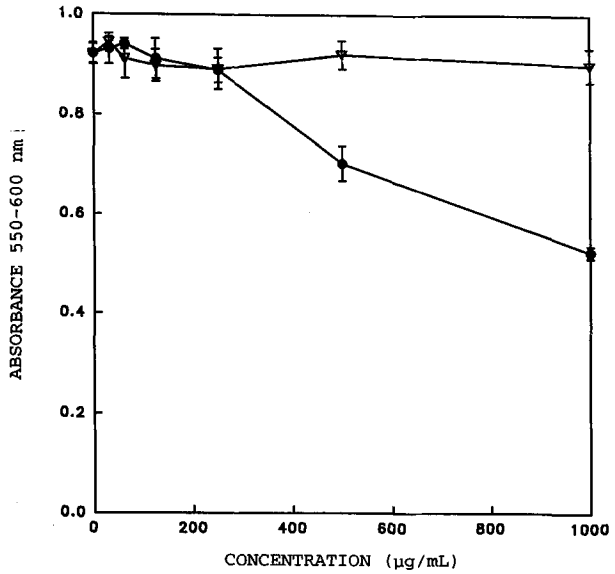


FIG. 5. Effect of tocotrienol-rich fraction (TRF) (●) and α-tocopherol (α-T) (▽) on the viability of MDA-MB-435 cells. MDA-MB-435 cells were incubated with various concentrations of TRF and α-T. 3,(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (25 μL) was added, and after 3 h, 100 μL of SDS extraction buffer was added and o.d. measurements made at 570 nm. Points are the average of mean values from three experiments ± SE.

We found that TRF markedly inhibited the proliferation of MDA-MB-435 human breast cancer cells in culture, having a concentration required to inhibit cell proliferation by 50% of 180 μg/mL, whereas α-T had no effect at concentrations up to 1000 μg/mL. We also found that in testing the compounds on the growth of these cells over a period of ten days, TRF inhibited the growth by 50% at 180 μg/mL (Fig. 3A) and completely inhibited growth at 500 μg/mL (Fig. 3B). Also, these cells were viable in the presence of TRF at concentrations up to 200 μg/mL, and they were viable at all concentrations used for α-T (Fig. 5). The ability of the cells to form colonies was not altered with α-T, but TRF inhibited the PE (Fig. 4). Because TRF in palm oil is made up of 30% α-T and because in our study α-T appeared to have no effect at the same concentration, this inhibition is likely due to the presence of tocotrienols.

Our present results confirm the findings of Komiyama and Yamaoka (12), who showed growth inhibition of human and mouse tumor cells (H69, HeLa, and P388) when the cells were exposed to tocotrienols for 72 h *in vitro*. Tocopherols, on the other hand, showed no significant effect. Also, vitamin E (α-T) supplementation alone has been ineffective in inhibiting the development of mammary carcinomas in rats treated with carcinogenic chemicals (26–28). In addition, initiation of colon tumors induced by the carcinogen, dimethylhydrazine, and mammary tumors induced by DMBA, were increased by feeding diets deficient in vitamin E tocopherols (29,30). Horvath and Ip (31), however, presented evidence that α-T, although ineffective by itself, was able to potentiate the ability of selenium to inhibit the development of mammary carcinomas induced by DMBA in female rats fed a high-fat diet. Tocotrienols, on the other hand, have been shown to inhibit the increase in plasma γ-glutamyltranspeptidase activity, a recognized marker of early neoplasia in rats treated with the hepatocarcinogen, 2-acetylaminofluorene (32). Survival of mice receiving transplanted tumor cells was also increased if they were fed tocotrienol in a dose-responsive way (11).

The exact reasons for the higher sensitivity of the breast cancer cells to tocotrienols are unknown, but the following possibilities can be suggested: (i) Tocotrienols may more easily cross the cell membrane, and (ii) they may undergo slow degradation within the cell. These observations indicate that tocotrienols merit further investigation as possible agents for breast cancer prevention and/or treatment.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Palm Oil Research Institute of Malaysia and in part by a grant to A.F.C. from the National Cancer Institute of Canada.

REFERENCES

1. Sylvester, P.W., Russell, M., Ip, M.M., and Ip, C. (1986) Comparative Effects of Different Animal and Vegetable Fats Before and During Carcinogenesis Administration on Mammary Tu-

morigenesis, Sexual Maturation and Endocrine Function in Rats, *Cancer Res. 46*, 757–762.
2. Sundram, K., Khor, H.T., Ong, A.S.H., and Pathmanathan, R. (1989), Effect of Dietary Palm Oils in Mammary Carcinogenesis in Female Rats Induced by 7,12-Dimethylbenz(α)anthracene, *Cancer Res. 49*, 1447–1451.
3. Kritchevsky, D., Weber, M.M., and Klurfeld, D.M. (1992) Influence of Different Fats (soybean oil, palm olein or hydrogenated oil) on Chemically-Induced Mammary Tumors in Rats, *Nutr. Res. 12*, S175–S179.
4. Hubbard, N.E., and Erickson, K.L. (1987) Influence of Dietary Fats on Cell Populations of Line Mouse Mammary Tumors; A Morphometric and Ultrastructural Study, *Cancer Lett. 35*, 281–294.
5. Hopkins, G.J., and Carroll K.K. (1979) Relationship Between Amount and Type of Dietary Fat in Promotion of Mammary Carcinogenesis Produced by 7,12-Dimethylbenz(α)anthracene, *J. Natl. Cancer Inst. 62*, 1009–1012.
6. Clinton, S.K., Mulloy, A.L., and Visek, W.J. (1984) Effects of Dietary Saturation on Prolactin Secretion, Carcinogen Metabolism and Mammary Carcinogenesis in Rats, *J. Nutr. 114*, 1630–1639.
7. Gammal, E.B., Carroll, K.K., and Plunkett. E.R. (1967) Effects of Dietary Fat on Mammary Carcinogenesis by 7,12-Dimethylbenz-α-anthracene in Rats, *Cancer Res. 27*, 1737–1742.
8. Rose, D.P., and Connolly, J.M. (1989) Stimulation of Growth of Human Breast Cancer Cell Lines in Culture by Linoleic Acid, *Biochem. Biophys. Res. Comm. 164*, 277–283.
9. Nesaretnam, K., Khor, H.T., Ganeson, J., Chong, Y.H., Sundram, K., and Gapor, A. (1992) The Effect of Vitamin E Tocotrienols from Palm Oil on Chemically Induced Mammary Carcinogenesis in Female Rats, *Nutr. Res. 12*, 879–892.
10. Tan, B. (1992) Antitumor Effects of Palm Carotenes and Tocotrienols in HRS/J Hairless Female Mice, *Nutr. Res. 12*, S163–S173.
11. Kato, A., Yamaoka, M., Tanaka, A., Komiyama, K., and Umezawa, I. (1985) Physiological Effect of Tocotrienols, *Yakugaku Zasshi 34*, 375–376.
12. Komiyama, K., and Yamaoka, M. (1989) Studies on the Biological Activity of Tocotrienols, *Chem. Pharm. Bull. 37*, 1369–1371.
13. Gould, M.N., Haag, J.D., Kennan, W.S., Tanner, M.A., and Elson, C.E. (1991) A Comparison of Tocopherol and Tocotrienol for the Chemoprevention of Chemically Induced Rat Mammary Tumors, *Am. J Clin. Nutr. 53*, 1068s–1070s.
14. Yamaoka, M., and Carrillo, M.J.H. (1990) Effect of Tocopherols and Tocotrienols on the Physicochemical Property of the Liposomal Membrane in Relation to Their Antioxidant Activity, *Chem. Phys. Lipids 55*, 295–300.
15. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) Free Radical Recycling and Intermembrane Mobility in the Antioxidant Properties of α-Tocopherol and α-Tocotrienol, *Free Radic. Biol. Med. 10*, 263–275.
16. Lathia, D., Braasch, A., and Theissen, U. (1988) Inhibitor Effects of Vitamin C and E on *In Vitro* Formation of *N*-Nitrosamine Under Physiological Conditions, *Front. Gastrointest. Res. 14*, 151–156.
17. Salomen, J.T., Salomen, R., and Lappetalainen, R. (1985) Risk of Cancer in Relation to Serum Concentrations of Selenium and Vitamin A and E, Match Case Control Analysis of Prospective Data, *Br. Med. J. 290*, 417–420.
18. Willet, W.C. (1986) Selenium, Vitamin E, Fiber and the Incidence of Human Cancer an Epidemiologic Perspective, *Adv. Exp. Med. Biol. 206*, 27–34.
19. Knekt, P., Aromaa, A., Maatela, J., Aaran, R.K., Nikkari, T., Hakama, M., Hakulinen, T., Peto, R., Saxen, E., and Teppo, L. (1988) Serum Vitamin E and Risk of Cancer Among Finnish Men During a Ten Year Follow-Up, *Am. J. Epidemiol. 127*, 28–41.
20. Knekt, P. (1988) Serum Vitamin E Level and Risk of Female Cancer, *Int. J. Epidemiol. 17*, 281–286.
21. Packer, L. (1991) Protective Role of Vitamin E in Biological System, *Am. J. Clin. Nutr. 53*, 1050S–1055S.
22. Sundram, K., and Gapor, A. (1992) A Vitamin E from Palm Oil Its Extraction and Nutritional Properties, *Lipid Technolog.*, November/December, 137–141.
23. Price, J.E., Polyzos, A., Zhang, R.D., and Daniels, L.M. (1990) Tumorigenicity and Metastasis of Human Breast Carcinoma Cell Lines in Nude Mice, *Cancer Res. 50*, 717–721.
24. Hansen, M.B., Nielsen, S.E., and Burg, K. (1989) Re-Examination and Further Development of a Precise and Rapid Dye Method for Measuring Cell Growth/Cell Kill, *J. Immunol. Methods 119*, 203–210.
25. Leth, T., and Sondergaard, H. (1977) Biological Activity of Vitamin E Compounds and Natural Materials by the Resorption-Gestation Test and Chemical Determination of the Vitamin E Activity in Foods and Feeds, *J Nutr. 107*, 2236–2243.
26. Wattenberg, L.W. (1972) Inhibition of Carcinogenic and Toxic Effects of Polycyclic Hydrocarbons by Phenolic Antioxidants and Ethoxyquin, *J. Natl. Cancer Inst. 48*, 1425–1430.
27. Dayton, S., Hashimoto, S., and Wollman, J. (1977) Effect of High-Oleic and High Linoleic Safflower Oils on Mammary Tumors Induced in Rats by 7,12-Dimethylbenzanthracene, *J. Nutr. 107*, 1353–1360.
28. Welsch, C.W. (1992) Relationship Between Dietary Fat and Experimental Mammary Tumorigenesis: A Review and Critique, *Cancer Res. 52*, 2040s–2048s.
29. Sumiyoshi, H. (1985) Effects of Vitamin E Deficiency on 1,2 Dimethylhydrazine Induced Intestinal Carcinogenesis in Rats, *Hiroshima J. Med. Sci. 34*, 363–369.
30. Ip, C. (1982) Dietary Vitamin E Intake and Mammary Carcinogenesis in Rats, *Carcinogenesis 3*, 1453–1456.
31. Horvath, P.M. and Ip, C. (1983) Synergistic Effect of Vitamin E and Selenium in the Chemoprevention of Mammary Carcinogenesis in Rats, *Cancer Res. 43*, 5335–5341.
32. Ngah, W.Z.W., Jarien, Z., San, M.M., Marzuki, A., Top, G.M., Shamaan, N.A., and Kadir, K.A. (1991) Effects of Tocotrienols on Hepatocarcinogenesis Induced by 2 Acetylaminofluorene in Rats, *Am. J. Clin. Nutr. 53*, 1076s–1081s.

[Received December 22, 1993, and in final revised form June 12, 1995; Revision accepted August 28, 1995]

Oxidative Susceptibility of Low Density Lipoprotein from Rabbits Fed Atherogenic Diets Containing Coconut, Palm, or Soybean Oils[1]

S.C. Yap[a], Y.M. Choo[a], N.F. Hew[b], S.F. Yap[c], H.T. Khor[d], A.S.H. Ong[e], and S.H. Goh[b,*]

[a]Palm Oil Research Institute of Malaysia, Departments of [b]Chemistry, [c]Pathology and [d]Biochemistry, University of Malaya, 59100 Kuala Lumpur, Malaysia, and [e]Malaysian Palm Oil Promotion Council, 50450 Kuala Lumpur, Malaysia

ABSTRACT: The oxidative susceptibilities of low density lipoproteins (LDL) isolated from rabbits fed high-fat atherogenic diets containing coconut, palm, or soybean oils were investigated. New Zealand white rabbits were fed atherogenic semisynthetic diets containing 0.5% cholesterol and either (i) 13% coconut oil and 2% corn oil (CNO), (ii) 15% refined, bleached, and deodorized palm olein (RBDPO), (iii) 15% crude palm olein (CPO), (iv) 15% soybean oil (SO), or (v) 15% refined, bleached, and deodorized palm olein without cholesterol supplementation [RBDPO(wc)], for a period of twelve weeks. Total fatty acid compositions of the plasma and LDL were found to be modulated (but not too drastically) by the nature of the dietary fats. Cholesterol supplementation significantly increased the plasma level of vitamin E and effectively altered the plasma composition of long-chain fatty acids in favor of increasing oleic acid. Oxidative susceptibilities of LDL samples were determined by Cu^{2+}-catalyzed oxidation which provide the lag times and lag-phase slopes. The plasma LDL from all palm oil diets [RBDPO, CPO, and RBDPO(wc)] were shown to be equally resistant to the oxidation, and the LDL from SO-fed rabbits were most susceptible, followed by the LDL from the CNO-fed rabbits. These results reflect a relationship between the oxidative susceptibility of LDL due to a combination of the levels of polyunsaturated fatty acids and vitamin E.

Lipids 30, 1145–1150 (1995).

Low density lipoprotein (LDL) has been identified as the principal proatherogenic lipoprotein (1,2). The accumulation of LDL-derived cholesterol in cells of the arterial wall is now widely believed to result from the chemical modification of LDL, especially the oxidatively modified LDL that has been detected in atherosclerotic lesions (3,4). Oxidized and mildly oxidized LDL, either wholly or the oxidized products leaving the particles, can have a number of deleterious effects, including chemotaxis for monocytes (5), inhibition of nitric oxide-mediated relaxation of coronary tone (6), and stimulation of inflammatory cell accumulation (7). Recognition and uncontrolled uptake of the oxidized LDL by macrophages lead to a chain of biochemical events which may result in the formation of foam cells, fatty streaks and, later, atherosclerosis.

Diet has been a major focus of research in relation to the environmental factors in cardiovascular disease. The development of atherosclerosis is an important aspect of cardiovascular disease and is influenced by plasma cholesterol levels (8). Plasma cholesterol levels are modulated by the amount and type of dietary fats and various micronutrients, such as tocotrienols (9,10). High levels of LDL-cholesterol are, however, not a sufficient condition for atherosclerosis, as the incidence of coronary heart disease among hypercholesterolemic individuals remains variable (11). This variation is a consequence of different biochemistry among individuals, of which modification and metabolism of LDL are probably important. Substantial and compelling evidence supports the hypothesis that oxidation of LDL plays an important role in atherogenesis (1,12). The inhibitory effect of antioxidants in the oxidation of human LDL is well documented (13), but there is still inadequate information on the influence of various fats with varying unsaturation on this process, or on the *in vivo* effects of antioxidants on LDL oxidation. The present paper examines the *ex vivo* oxidative susceptibilities of LDL derived from rabbits fed atherogenic diets containing palm oil as compared to diets containing coconut and soybean oils.

MATERIALS AND METHODS

Animals and diets. New Zealand white rabbits (six-month-old) were randomly divided into five groups and fed *ad libitum* semisynthetic diets (Table 1) containing 5% (w/w) commercial pellets, 95% (w/w) purified substances consisting of (i) 13% coconut oil plus 2% corn oil (CNO, n = 5), (ii) 15% refined, bleached and deodorized palm olein (RBDPO, n = 6), (iii) 15% crude palm olein (CPO, n = 4), or (iv) 15% soybean oil (SO, n = 6), all supplemented with cholesterol (5 g/kg), or

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, 1993.

*To whom correspondence should be addressed at the Department of Chemistry, University of Malaya, 59100 Kuala Lumpur, Malaysia.

Abbreviations: CNO, coconut oil with 13.3% corn oil; CPO, crude palm olein; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acids; RBDPO, refined, bleached, and deodorized palm olein; RBDPO(wc), refined, bleached, and deodorized palm olein without cholesterol supplementation; SO, soybean oil; α-TAc, α-tocopheryl acetate; VLDL, very low density lipoprotein.

Copyright © 1995 by AOCS Press

TABLE 1
Formulation of Semisynthetic Feed[a]

Component	Composition (g/kg)
Casein-VITAFREE	200
DL-Methionine	3
Dextrose monohydrate	150
Corn starch	240
CELUFIL (nonnutritive bulk)	200
AIN mineral mixture	45
AIN vitamin mixture	10
Choline dihydrogen citrate	2
Dietary fat	150

[a]Fifty grams of commercial-rabbit feed pellets, 950 g of a mixture of feed materials (from U.S. Biochemical Corp., Cleveland, OH) and dietary fats, and 5 g of cholesterol were blended and repelletized.

(v) RBDPO diet without supplementation of cholesterol [RBDPO(wc), n = 5]. All the synthetic diet components and cholesterol were purchased from United States Biochemical Corporation (Cleveland, OH). Blood samples were collected from overnight-fasted rabbits before and after a 12-wk dietary treatment.

Oxidation of LDL. LDL (density 1.019–1.063 g/mL) was isolated from plasma according to a modified method of Havel *et al.* (14), using sequential density ultracentrifugation in KBr solution at 4°C. The LDL was dialyzed at 4°C for 24 h against four changes of 50–100 vol of deoxygenated phosphate-buffered saline (0.01 M, pH 7.4). Dialyzed LDL samples were diluted with oxygen-saturated phosphate-buffered solution (0.01 M, pH 7.4) to 0.1 mg protein/mL and incubated at 37°C. Oxidation was initiated by addition of copper (II) chloride to a final concentration of 4.0 μM. Absorbance at 234 nm was continuously monitored by a U-200 Hitachi spectrophotometer (Tokyo, Japan) (15,16).

Analyses. Lipids from plasma and LDL were extracted by the method of Folch *et al.* (17). Plasma vitamin E content was analyzed by a modified method of Lang *et al.* (18) using normal-phase high-performance liquid chromatography equipped with a fluorescence detector. The plasma and LDL lipids were saponified with ethanolic potassium hydroxide containing 1 mg of butylated hydroxytoluene in vacuum-sealed tubes for 2 h at 100°C. After acidification, free fatty acids and methyl esters were extracted with hexane and methylated with boron trifluoride–methanol (19). The total fatty acid composition was analyzed using an HP5970A gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a 30-m 0.5-μm DB-23 capillary column from J&W Scientific (Folsom, CA).

Statistics. The Student's *t*-test was used for statistical evaluation of the results, and differences at $P < 0.05$ were considered significant.

RESULTS

The fatty acid compositions of various dietary fats in the diets are given in Table 2. The CNO-based diet is rich in saturated fatty acids (lauric, 12:0; and myristic, 14:0), the palm oil diets [RBDPO, CPO, and RBDPO(wc)] are rich in oleic (18:1) and palmitic (16:0) acids, and the SO diet is rich in polyunsaturated fatty acids, mainly linoleic acid (18:2). The compositions of intrinsic vitamin E components in the various diets are given in Table 3, with the palm and soybean oil diets having about the same total vitamin E content, whereas the coconut oil diet (CNO) has a slightly lower vitamin E content. Fatty acid profiles of the plasma and LDL derived from rabbits on the various diets are given in Tables 4 and 5. Substantial changes in the composition of long-chain fatty acids were observed. The levels of oleic acid for all rabbits on diets supplemented with cholesterol were seen to increase quite remarkably after a 12-wk dietary treatment, particularly in the palm oil groups (RBDPO and CPO), which showed significantly higher plasma and LDL oleic acid content than those from the CNO and SO groups. The SO-fed rabbits showed increases in linoleic acid levels in plasma and LDL which were about twofold higher than those found in other rabbits.

Supplementation with dietary cholesterol generally increased the yield of LDL which were required to carry out the oxidation experiments. The susceptibility of the LDL samples to Cu^{2+}-oxidation is reflected by the lag time and lag-phase slope observed in the plots of absorption at 234 nm vs. time (typical results are shown in Fig. 1). Data on lag times, lag-phase slopes, propagation phase, etc. are given in Table 6. LDL samples from palm oil groups were shown to have longer lag times than CNO and SO groups in the oxidation. As determined by the slope of the lag phase, the rates of oxidation of LDL derived from CNO and SO groups were shown to be about sixfold faster than LDL from the palm oil groups. However, during the propagation phase, the oxidation rate of the LDL from the SO group was about threefold higher than the other groups. The high absorption at the end of the oxidation for each sample of the SO group was indicative of a

TABLE 2
Fatty Acid Composition of the Dietary Fats (% of total fatty acids)[a]

Fat	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
CNO	3.4	5.1	4.6	40.4	16.4	8.8	—	3.0	9.7	8.6	—	—	—
RBDPO	—	—	—	0.2	0.9	36.1	0.2	3.8	46.8	11.2	0.5	0.2	—
CPO	—	—	—	0.2	1.0	38.3	0.2	4.1	45.0	11.0	0.4	0.3	—
SO	—	—	—	0.1	0.1	11.0	0.1	3.7	19.8	55.9	8.6	0.2	0.4

[a]CNO, coconut oil with 13.3% corn oil; RBDPO, refined, bleached, and deodorized palm olein; CPO, crude palm olein; and SO, soybean oil.

TABLE 3
Composition of the Vitamin E Components in Various Diets[a]

Diet	Vitamin E (mg/kg diet)							
	α-TAc	α-T	α-T_3	γ-T	γ-T_3	δ-T	δ-T_3	Total
CNO	176	13	0	2	0	0	1	192
RBDPO	176	20	14	0	22	0	8	240
CPO	176	22	15	0	21	0	10	244
SO	176	2	0	36	0	12	0	226
RBDPO(wc)	176	18	13	0	20	0	8	235

[a]Abbreviations as in Table 2. RBDPO(wc) is RBDPO-based diet but without cholesterol. The weight of *dl*-α-tocopheryl acetate (α-TAc) is given as α-T equivalent; T, tocopherol; T_3, tocotrienol.

TABLE 4
Fatty Acid Composition of Plasma Lipids (% of total fatty acids)[a]

Group	n	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Baseline	—	0.8 ± 0.4	6.3 ± 2.1	46.7 ± 6.0	1.4 ± 2.0	10.3 ± 2.0	18.4 ± 4.1	14.2 ± 4.0	1.3 ± 1.0	0.1 ± 0.1
CNO	5	1.5 ± 0.6	6.3 ± 3.5	29.5 ± 3.2^b	3.7 ± 1.2	6.8 ± 1.2	33.7 ± 3.3^b	18.9 ± 1.4^b	0.4 ± 0.2	0.2 ± 0.1
RBDPO	6	0.1 ± 0.1	2.2 ± 0.2	30.2 ± 2.8^b	4.2 ± 1.7	5.7 ± 1.2	43.7 ± 3.9^c	13.6 ± 6.4^b	0.3 ± 0.1	0.2 ± 0.1
CPO	4	0.1 ± 0.1	1.2 ± 1.0	31.6 ± 3.9^b	3.7 ± 0.8	5.8 ± 1.5	44.5 ± 5.4^c	12.7 ± 2.4^b	0.2 ± 0.1	0.2 ± 0.1
SO	6	0.2 ± 0.1	2.1 ± 0.9	20.8 ± 2.8^c	3.4 ± 1.1	5.9 ± 1.3	32.5 ± 5.1^b	33.2 ± 2.4^c	1.0 ± 0.5	0.3 ± 0.2
RBDPO(wc)	5	0.6 ± 0.3	9.7 ± 3.2	44.8 ± 8.8^d	0.6 ± 0.1	5.3 ± 1.4	27.8 ± 3.0^b	10.8 ± 1.9^b	0.3 ± 0.1	0.1 ± 0.1

[a]Baseline data is the average value for rabbits fed on commercial feed before switching to atherogenic diets. Abbreviations as in Tables 2 and 3. n, Number of rabbit in each group

[b–d]Means in the same column with different superscripts are significantly different ($P < 0.05$).

TABLE 5
Fatty Acid Composition of Low Density Lipoproteins (% of total fatty acids)[a]

Group	n	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
CNO	5	1.7 ± 0.4	7.0 ± 0.7	39.8 ± 2.3	2.2 ± 1.8	7.7 ± 1.1	28.6 ± 2.6	11.9 ± 1.1^b	0.4 ± 0.5	1.4 ± 0.8
RBDPO	6	1.0 ± 0.6	3.7 ± 0.6	37.7 ± 2.4	3.4 ± 1.7	5.9 ± 1.1	36.9 ± 0.8	12.2 ± 2.1^b	0.3 ± 0.4	0.8 ± 0.2
CPO	4	0.3 ± 0.1	4.5 ± 1.0	33.5 ± 2.9	5.4 ± 0.8	4.3 ± 0.3	43.7 ± 2.5	8.0 ± 1.9^b	0.2 ± 0.1	0.2 ± 0.1
SO	6	0.6 ± 0.4	1.2 ± 0.1	37.4 ± 2.5	2.6 ± 2.3	8.6 ± 1.3	29.8 ± 3.5	21.2 ± 4.5^c	0.6 ± 0.1	0.6 ± 0.1
RBDPO(wc)	5	0.3 ± 0.1	7.2 ± 2.9	46.3 ± 5.3	3.7 ± 1.3	4.0 ± 1.0	27.7 ± 1.5	10.3 ± 0.1^b	0.2 ± 0.1	0.2 ± 0.2

[a]Abbreviations as in Tables 2 and 3. Values are means ± SD. n, Number of rabbits in each dietary group.

[b,c]Means in the same column with different superscripts are significantly different ($P < 0.05$).

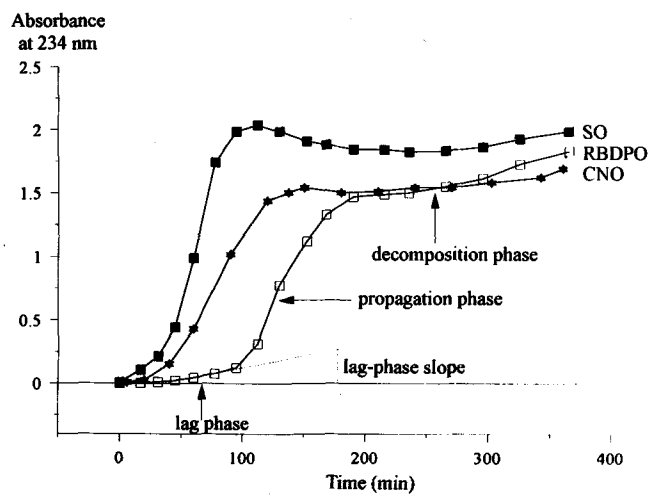


FIG. 1. Time course of absorption at 234 nm for the Cu^{2+}-catalyzed oxidation of low density lipoprotein isolated from the rabbits fed atherogenic diets containing coconut oil and corn oil (CNO); refined, bleached, and deodorized palm olein (RBDPO), and soybean (SO).

larger amount of conjugated dienes formed from the high content of polyunsaturated fatty acids (PUFA).

DISCUSSION

The dietary regimes used provided three diverse types of dietary fats, i.e., highly saturated (coconut), moderately saturated, and monounsaturated (palm), as well as highly polyunsaturated (soybean) fatty acids. The fatty acid distributions of the diets (Table 2) have undergone considerable modulation by the time they are incorporated into the LDL particles, so that their distribution in plasma only partially reflects the nature of fatty acids of the diets (Tables 4,5). The PUFA levels of the LDL particles increased noticeably with the SO-diet; those with the CNO and palm oil [i.e., RBDPO, CPO, and RBDPO(wc)] diets remained virtually unchanged. Despite the fact that the CNO diet contains mainly medium-chain fatty acids, the incorporation of these fatty acids in the LDL is minimal, a reflection of the different metabolic pathway for the handling of short- and medium- chain fatty acids as compared to the long-chain fatty acids.

TABLE 6
Effects of the Levels of Polyunsaturated Fatty Acids (PUFA) and Vitamin E on the Oxidative Susceptibility of Low Density Lipoproteins[a]

Dietary group	n	Lag time (min)	Lag-phase slope ($\times10^{-4}$/min)	Propagation-phase slope $\times10^{-3}$/min	Maximum absorbance at 234 nm	Vitamin E (μM)	PUFA (%)
CNO	5	56 ± 6^b	20.0 ± 9.0	15 ± 4	1.52 ± 0.35	38 ± 0.14^b	12.7 ± 2.4^b
RBDPO	6	111 ± 23^c	2.9 ± 0.9	19 ± 4	1.35 ± 0.08	41 ± 31^b	13.3 ± 2.7^b
CPO	4	103 ± 25^c	2.3 ± 1.3	15 ± 6	1.19 ± 0.02	59 ± 25^b	8.4 ± 2.1^b
SO	6	54 ± 17^b	18.0 ± 7.0	45 ± 4	2.18 ± 0.10	40 ± 16^b	22.4 ± 4.7^c
RBDPO(wc)	5	110 ± 8^c	3.5 ± 0.5	19 ± 5	1.26 ± 0.17	8.8 ± 3.7^c	10.7 ± 0.4^b

[a]Abbreviations as in Tables 2 and 3. Values are means ± SD. n, Number of rabbits in each dietary group. Lag-phase slope (absorbance unit/min) is the increase of absorbance at 234 nm with time during the lag phase. Propagation-phase slope (absorbance unit/min) is the increase of absorbance at 234 nm with time during the propagation phase.
[b,c]Means in the same column with different superscripts indicate differences are statistically significant ($P < 0.05$).

Although the actual initiation processes of the peroxidation of LDL are not precisely known, accumulated evidence indicates that the end products are from lipid peroxidation. Oxidation studies using *in vitro* experiments have suggested that the properties of LDL particles oxidized *in vitro* may be similar to those purported to arise *in vivo* (20). Oxidative stability of LDL particles can be conveniently studied using the Cu^{2+}-catalyzed oxidation established by Esterbauer *et al.* (16). The main targets of this oxidation are the PUFA (mainly linoleic, but also include, to a small extent, linolenic and arachidonic acids), and the formation of conjugated diene hydroperoxides (absorption at 234 nm) provides a kinetic profile containing lag, propagation, and decomposition phases (as shown in Fig. 1). It is noted that because this is a lipid peroxidation process, the inhibition of autoxidation in LDL can occur by antioxidants such as α-tocopherol, carotenoids, ubiquinol, etc. After the depletion of the antioxidants, a rapid oxidation occurs (propagation phase), and finally, a plateau is reached (decomposition phase) when the breakdown of peroxides occurs. The lag time (and also the lag-phase slope) which precedes the onset of rapid lipid autoxidation is important as it reflects the induction period of radical oxidation and is an indicator of the oxidative susceptibility of the LDL.

The results shown in Figure 1 and Table 6 provide comparisons of the lag times and lag-phase slopes for the oxidation of LDL resulting from the three dietary fats. The lag times and lag-phase slopes were reflective of the dietary fats, with the LDL from rabbits fed palm oil diets having the highest oxidative stability. Previous *in vitro* work (21) has indicated that increasing levels of antioxidants (vitamin E, ubiquinols, carotene, etc.) can effectively prolong lag times. The present data (CNO vs. CPO, $P < 0.1$) appear to support the observation that the vitamin E concentration is an important factor in determining the oxidative stability of LDL derived from rabbits fed with various types of dietary fats; LDL samples from rabbits supplemented with vitamin E or C are actually more stable than LDL obtained from rabbits deprived of these antioxidants in their diet (Goh, S.H., and Hew, N.F., unpublished data). However, there may be other antioxidant components, e.g., ubiquinols (Choo, Y.M., and Hazura, H., unpublished data), in palm oil which may be important but have not yet been considered. Tocotrienols may not be directly involved in the inhibition of oxidation as they are present in very low concentrations in LDL. Although α-tocopherol is selectively accumulated in the plasma and liver (22), the tocotrienols appear not to be, and apparently are excreted. However, tocotrienols are expected to remain as lipid antioxidants throughout the processes of ingestion, absorption, and transport in chylomicrons and very low density lipoproteins (VLDL). The plasma vitamin E level is generally low in normal rabbits but increased with cholesterol feeding, as noted in previous reports (22). This may be due to the cholesterol-induced increase in formation of various lipoproteins which are carriers of vitamin E. Vitamin E present in LDL is probably insufficient to effectively protect against oxidation of all the PUFA (23). Results from the CPO diet, which is unrefined liquid palm oil, are also noteworthy in that although the oil is rich in carotenes in addition to vitamin E (in contrast to RBD palm olein which lacks carotenes), the carotenes in the diet did not confer increased stability to the LDL. This is due to the fact that only small amounts of carotenes are found in the plasma (or LDL) and are metabolized rapidly to vitamin A.

Although many studies have focused on the oxidation of LDL, not much is known about the actual chemistry of oxidation of lipoproteins under *in vivo* conditions. Recent work (24) has indicated that without vitamin C, vitamin E is not only insufficient to protect LDL against oxidation, but may even be prooxidant under certain conditions. Other minor antioxidants, notably ubiquinols, are more effective antioxidants in aqueous lipid emulsions. Furthermore, there is considerable heterogeneity among lipoproteins, and recent evidence indicates that high density forms of LDL and lipoprotein(a) may be more atherogenic (25). The observed relationship between the oxidative stability of LDL and PUFA and vitamin E levels is very likely indicative of the presence of preformed or endogenous peroxides in the LDL. It is believed that oxidative damage of lipids in LDL may have occurred in its precursor particles (e.g., chylomicrons and VLDL), in which case the presence of the various types of dietary antioxidants will be beneficial.

Whereas levels of antioxidants may be an important factor in LDL oxidative stability, we have found that the degree of polyunsaturation of the LDL is also important. The propagation phase for the oxidation of LDL from SO-rabbits is much more rapid and is directly dependent on the LDL PUFA content. In terms of the degree of polyunsaturation, the CNO-diet (because corn oil was also added to provide essential fatty acids) has a slightly higher ratio of PUFA compared to palm oil diets if the medium- and short-chain (≤C12) fatty acids are neglected. The LDL from CNO rabbits show essentially similar lag- phase slopes for those of SO-rabbits, but much higher ones than those from the various palm oil diet groups. The shorter lag times and larger lag-phase slopes for LDL from rabbits fed coconut oil and SO diets are apparently the result of oxidative stress which is influenced by high PUFA content and low vitamin E levels. The tendency for rapid and extensive oxidation (highest absorbance) of LDL from the SO-diet would be theoretically an undesirable feature, as oxidized LDL has been demonstrated to be extensively taken up by macrophages. Present results support the previous report (26) that the monounsaturated oleic acid offers oxidative resistance to LDL similar to saturated fatty acids. The LDL of rabbits fed atherogenic (RBDPO) and nonatherogenic [RBDPO(wc)] palm oil diets show similar oxidative stability. Aside from the presence of similar vitamin E levels, this may be chiefly attributed to their unique fatty acid profiles, in that palmitic and oleic acids constituted about 75% of the total fatty acids.

Although coronary heart disease and atherosclerosis have generally been linked to excessive consumption of cholesterol and some saturated fats, the oxidative resistance of LDL and/or its protection are likely to be equally important factors for consideration in dietary recommendations. For example, epidemiological studies (27) have suggested that antioxidants in the plasma are associated with lower rates of cardiovascular disease. In many countries where relatively high fat/cholesterol diets are not correlated with high mortality from cardiovascular disease, there is usually a high consumption of vegetables and fruits which provides natural antioxidants. The "French paradox," an apparent compatibility of a high-fat diet with low incidence of coronary atherosclerosis, has been attributed to phenolic antioxidants from red wine (28) apart from a high consumption of fruits and vegetables. In view of the suggested importance of oxidative stability of LDL, adequate amounts of dietary antioxidants and a high monounsaturated fat diet with moderate amounts of saturated and polyunsaturated fat may be optimal.

ACKNOWLEDGMENTS

This project was supported by P.O.R.I.M. and the Malaysian IRPA grants.

REFERENCES

1. Steinbrecher, U.P., Zhang, H., and Longheed, M. (1990) Role of Oxidatively Modified LDL in Atherosclerosis, *Free Rad. Biol. Med. 9*, 155–168.
2. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witztum, J.L. (1989) Beyond Cholesterol: Modifications of Low-Density Lipoprotein that Increase Its Atherogenicity, *New Engl. J. Med. 320*, 915–924.
3. Witztum, J.L., and Steinberg, D. (1991) Role of Oxidized Low Density Lipoprotein in Atherogenesis, *J. Clin. Invest. 88*, 1785–1792.
4. Boyd, H.C., Gown, A.M., Wolfbauer, G., and Chait, A. (1989) Direct Evidence for a Protein Recognised by a Monoclonal Antibody Against Oxidatively Modified LDL in Atherosclerotic Lesions from a Watanabe Heritable Hyperlipidemic Rabbit, *Am. J. Pathol. 135*, 815–825.
5. Quinn, M.T., Parthasarathy, S., Fong, L.G., and Steinberg, D. (1987) Oxidatively Modified Low Density Lipoproteins: A Potential Role in Recruitment and Retention of Monocyte/Macrophages During Atherogenesis, *Proc. Natl. Acad. Sci. USA 84*, 2995–2998.
6. Stam, H., Hülsman, J.F., Jongkind, J.F., Van der Kraaij, A.M.M., and Koster, J.F. (1989) Endothelial Lesions, Dietary Composition and Lipid Peroxidation, *Eicosanoids 2*, 1–14.
7. Rosenfeld, M.E., Palinski, W., Yla-Herttuala, S., and Carew, T.E. (1990) Macrophages, Endothelial Cells, and Lipoprotein Oxidation in the Pathogenesis of Atherosclerosis, *Toxicol. Pathol. 18*, 560–571.
8. Thompson, G. (1992) Progression and Regression of Coronary Artery Disease, *Current Opinion Lipidology 3*, 263–267.
9. Tan, D.T.S., Khor, H.T., Low, W.H.S., Ali, A., and Gapor, A. (1991) Effect of a Palm-Oil-Vitamin E Concentrate on the Serum Lipoprotein Lipids in Humans, *Am. J. Clin. Nutr. 53*, 1027S–1030S.
10. Watkins, T., Lenz, P., Gapor, A., Struck, M., Tomeo, A., and Bierenbuam, M. (1993) γ-Tocotrienol as a Hypocholesterolemic and Antioxidant Agent in Rats Fed Atherogenic Diets, *Lipids 28*, 1113–1118.
11. Holme, I., Enger, S.C., Helgeland, A., Hjermann, I., Leren, P., Lund-Larsen, P.G., Solberg, L.A., and Strong, J.P. (1981) Risk Factors and Raised Atherogenic Lesions in Coronary and Cerebral Arteries. Statistical Analysis from the Oslo Study, *Arteriosclerosis 1*, 250–256.
12. Ledwozyw, A., Michalak, J., Stepien, A., and Kadziolka, A. (1986) The Relationship Between Plasma Triglycerides, Cholesterol, Total Lipids and Lipid Peroxidation Products During Human Atherosclerosis, *Clin. Chim. Acta 155*, 275–284.
13. Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Striegl, G., and Jurgens, G. (1990) Biochemical, Structural, and Functional Properties of Oxidized Low-Density Lipoprotein, *Chem. Res. Toxicol. 3*, 77–92.
14. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) The Distribution and Chemical Composition of Ultracentrifugally Separated Lipoproteins in Human Serum, *J. Clin. Invest. 34*, 1345–1353.
15. Quehenberger, O., Koller, E., Jürgens, G., and Esterbauer, H. (1987) Investigation of Lipid Peroxidation in Human Low Density Lipoprotein, *Free Rad. Res. Comm. 3*, 233–242.
16. Esterbauer, H., Striegl, G., Puhl, H., and Rotheneder, M. (1989) Continuous Monitoring of *in vitro* Oxidation of Human Low Density Lipoprotein, *Free Rad. Res. Comm. 6*, 67–75.
17. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
18. Lang, J.K., Gohil, K., and Packer, L. (1986) Simultaneous Determination of Tocopherols, Ubiquinols, Ubiquinones in Blood, Plasma, Tissue Homogenates, and Subcellular Fractions, *Anal. Biochem. 157*, 106–116.
19. Blaton, V.H., and Peeters, H. (1972) Integrated Approach to Plasma Lipid and Lipoprotein Analysis, *Blood Lipids Lipoproteins: Quantitation, Composition and Metabolism* (Nelson, G.J., ed.) pp. 275–314, Wiley Interscience, New York.
20. Steinbrecher, U.P., Witztum, J.L., Parthasarathy, S., and Steinberg, D. (1987) Decrease in Reactive Amino Groups During Oxidation or Endothelial Cell Modification of LDL. Correlation

with Changes in Receptor-Mediated Catabolism, *Arteriosclerosis 7*, 135–143.
21. Esterbauer, H., Rotheneder, M., Striegl, G., Waeg, G., Ashy, A., Sattler, W., and Jurgens, G. (1989) Vitamin E and Other Lipophilic Antioxidants Protect LDL Against Oxidation, *Fat Sci. Technol. 91*, 316–324.
22. Hew, N.F., Khor, H.T., Choo, Y.M., Yap, S.F., and Goh, S.H. (1994) Unusual Accumulation of α-Tocopherol in Rabbits Fed Palm-Oil Diets Enhanced with Various Vitamin E Components, *Malaysian J. Sci. 15A*, 73–78.
23. Esterbauer, H., Jürgens, G., Quehenberger, O., and Koller, E. (1987) Autoxidation of Human Low Density Lipoprotein: Loss of Polyunsaturated Fatty Acids and Vitamin E and Generation of Aldehydes, *J. Lipid Res. 28*, 495–509.
24. Ingold, K.U., Bowry, V.W., Stocker, R., and Walling, C. (1993) Autoxidation of Lipids and Antioxidation by α-Tocopherol and Ubiquinol in Homogeneous Solution and in Aqueous Dispersions of Lipids: Unrecognized Consequences of Lipid Particle Size as Exemplified by Oxidation of Human Low Density Lipoprotein, *Proc. Natl. Acad. Sci. USA 90*, 45–49.
25. Chait, A., Brazg, R.L., Tribble, D.L., and Krauss, R.M. (1993) Susceptibility of Small, Dense, Low-Density Lipoproteins to Oxidative Modification in Subjects with the Atherogenic Lipoprotein Phenotype, Pattern B, *Am. J. Med. 94*, 350–356.
26. Parthasarathy, S., Khoo, J.C., Muller, E., Barnett, J., Witztum, J.L., and Steinberg, D. (1990) Low Density Lipoprotein Rich in Oleic Acid Is Protected Against Oxidative Modification: Implications for Dietary Prevention of Atherosclerosis, *Proc. Natl. Acad. Sci. USA 87*, 3894–3898.
27. Rimm, E.B., Stampfer, M.J., Ascherio, A., Giovannucci, E., Colditz, G.A., and Willet, W.C. (1993) Vitamin E Consumption and the Risk of Coronary Heart Disease in Men, *New Engl. J. Med. 328*,1450–1456.
28 Frankel, E.N., Kanner, J., German, J.B., Parks, E., and Kinsella, J.E. (1993) Inhibition of Oxidation of Human Low-Density Lipoprotein by Phenolic Substances in Red Wine, *The Lancet 341*, 454–456.

[Received November 17, 1993, and in final revised form June 23, 1995; Revision accepted July 30, 1995]

Dietary Lipid Modification of Myocardial Eicosanoids Following Ischemia and Reperfusion in the Rat[1]

Mahinda Y. Abeywardena* and John S. Charnock

CSIRO, Division of Human Nutrition, Glenthorne Laboratory, O'Halloran Hill, SA 5158, Australia

ABSTRACT: Several different edible oils were compared for their ability to modify eicosanoid biosynthesis following experimentally-induced myocardial ischemia and reperfusion in the rat. Two types of palm oil [neutralized, bleached, and deodorized (NBDPO) and refined, bleached, and deodorized (RBDPO)] and partially hydrogenated soybean oil (SBO) were tested against a diet supplemented with sunflower seed oil (SSO) rich in n-6 polyunsaturated fatty acids (PUFA). Fish oil (FO) rich in n-3 PUFA, with its known cardioprotective actions, served as an internal reference point for the study. Test oils were fed as a 12% (w/w) supplement for nine months before the induction of myocardial ischemia and reperfusion. Palm oil diets exerted effects indistinguishable from the SBO group against cardiac arrhythmia, which occurred following alterations to coronary blood flow. Arrhythmic potentials, as expressed by a hierarchical scale (0–9) of arrhythmia score, were: SSO, 1.5 ± 0.5; FO, 0.9 ± 0.4; SBO, 3.1 ± 0.5*; NBDPO, 3.2 ± 0.5*; RBDPO, 3.3 ± 0.6*; *$P < 0.05$ vs. SSO. Following ischemia and reperfusion, both SSO and RBDPO groups tended to show an increase in myocardial prostacyclin, with the effect being more prominent in the RBDPO group (SSO, 10%; RBDPO, 25%). Thromboxane production was reduced in the FO group. Interestingly, cardiac muscle from both FO and palm oil groups displayed a reduced capacity to produce 12-hydroxyeicosatetraenoic acid SSO, 591.9 ± 95.8; SBO, 375.5 ± 48.9; NBDPO, 287.2 ± 64.7*; RBDPO, 230.9 ± 80.2**; FO, 203.7 ± 81.4** (ng/g dry wt, *$P < 0.05$, **$P < 0.01$). No clear relationship was seen between the availability of 20:4n-6 in myocardial phospholipids and eicosanoid profile. Data suggests that fatty acid composition of edible oils is not the only determinant of arrhythmic vulnerability and eicosanoid production.

Lipids 30, 1151–1156 (1995).

Although the influence of dietary fat on the pathogenesis of coronary heart disease has been recognized for several decades (1), direct evidence for the modification of arrhythmic vulnerability by dietary fatty acids has emerged only recently (2). For example, findings in this laboratory identify saturated animal fat as proarrhythmic, while either n-3 or n-6 polyunsaturated fatty acids (PUFA) possess cardioprotective actions in several whole animal models of sudden cardiac death (3,4). Interestingly, dietary monounsaturated fatty acids, such as olive oil, have been found to exert an effect similar to those of saturated fat following experimentally-induced myocardial ischemia and reperfusion (5). These observations therefore tend to implicate the fatty acid profile of edible oils and fats as a key determinant of arrhythmic vulnerability.

The biochemical basis for dietary lipid modification of arrhythmogenesis has not yet been identified with certainty as many factors influence cardiac rhythm, and because the immediate cause may vary with the type of arrhythmic stimulus (6,7). Nevertheless, changes in the myocardial production of eicosanoids are thought to be central to these effects as this group of lipid mediators represents a key regulatory mechanism in the cardiovascular system (7,8). Furthermore, their production is influenced by changes in the availability of precursor/inhibitor fatty acids in the cell (9). Therefore, changes in the composition of the myocardial membranes through dietary means may influence cardiovascular function due to modifications in the endogenous eicosanoid profile. Indeed, recent findings from this laboratory indicate that antiarrhythmic actions of dietary fish oil (FO) may be mediated through an eicosanoid-related mechanism (10).

In the present study, we have compared the effect of long-term (nine month) feeding of several different edible oils having variable fatty acid profiles on eicosanoid metabolism in rats after experimentally-induced brief regional myocardial ischemia and reperfusion. The experimental protocol employed in the present study also allowed an investigation of the vulnerability to and severity of cardiac arrhythmias, which originate in consequence to changes in coronary blood flow, to be made in these animals. A partially hydrogenated soybean oil (SBO) preparation and two different types of palm oils—refined, bleached, and deodorized (RBDPO); and neutralized, bleached, and deodorized (NBDPO)—were compared against sunflower seed oil (SSO) that was rich in linoleic acid (18:2n-6). A diet rich in n-3 PUFA of marine origin (MaxEPA, R.P. Scherer Holdings, Ltd., Melbourne, Australia) that has been reported to possess potent cardioprotec-

[1]Based on a paper presented at the PORIM International Palm Oil Congress, Kuala Lumpur, Malaysia, September 1993.

*To whom all correspondence should be addressed at CSIRO, Division of Human Nutrition, P.O. Box 10041, Gouger Street, Adelaide SA 5000, Australia.

Abbreviations: AA, arachidonic acid; A.S., arrhythmia score; EPA, eicosapentaenoic acid; FO, fish oil; 12-HETE, 12-hydroxyeicosatetraenoic acid; LO, lipoxygenase; NBDPO, neutralized, bleached, and deodorized palm oil; PGI_2, prostacyclin; PUFA, polyunsaturated fatty acids; RBDPO, refined, bleached, and deodorized palm oil; SBO, partially hydrogenated soybean oil; SSO, sunflower seed oil; TxA_2, thromboxane A_2; VF, ventricular fibrillation; VPB, ventricular premature beats; VT, ventricular tachycardia.

Copyright © 1995 by AOCS Press

tive qualities (2–5) also was included as a positive control for the study.

MATERIALS AND METHODS

Animals and diets. Weanling male rats of the hooded Wistar type were maintained on standard rat chow for four weeks and then divided into five experimental groups (n = 18 per group) and fed different lipid-supplemented diets for nine months. The preparation and storage of various diets and the feeding protocol were as reported previously (11,12). In brief, standard rat diets (Milling Industries, Adelaide, Australia) were supplemented (12% w/w) with the respective dietary oil, which included SSO; NBDPO; RBDPO; SBO, and purified FO (MaxEPA® c.f.). Soybean oil was only partially hydrogenated to yield melting characteristics similar to that of palm oil. SSO was from Nuttelex Ltd. (Victoria, Australia), and palm oils and SBO were obtained from the Palm Oil Research Institute of Malaysia (Bandar Baru Bangi, Malaysia). Standard rat diet and the lipid-supplemented diets were routinely analyzed for the total fat content, peroxide value, and the fatty acid composition (11–13). In addition, each new batch of the standard rat diet was fully analyzed for protein, fat, fiber, and vitamin levels, as well as for major trace elements, by an independent laboratory (Australian Government Analytical Laboratory, Adelaide, Australia) in order to minimize batch variations. Animals were housed in stainless-steel wire cages with free access to food and water and cared for according to the National Health and Medical Research Council (Canberra, Australia) and CSIRO (Canberra, Australia) guidelines for experimentation in animals.

Lipid analyses. Lipids were extracted with chloroform/methanol (2:1, vol/vol, containing 0.01% butylated hydroxytoluene as antioxidant), and were purified as described previously (12,13). Fatty acid methyl esters were prepared by treatment with sodium methoxide and were further purified using silicic acid columns (Biosil A; BioRad Laboratories, Richmond, CA). A gas chromatograph (HP 5890), equipped with a 30-m long megabore column (i.d. 0.53 mm, DB-23; J & W Scientific, Folsom, CA) was used for the analysis of fatty acid methyl esters.

Myocardial ischemia and reperfusion. The procedure is similar to that previously described in detail (3). Overnight fasted animals were anesthetised with pentobarbitone sodium (60 mg/kg, i.p.) and artificially ventilated before thoracotomy and opening of the chest cavity. The heart was exteriorized and a loose silk ligature placed around the left anterior descending coronary artery (3,5). After a 15-min stabilization period, regional myocardial ischemia was induced (5 min) by tightening the ligature. Arrhythmias induced by reperfusion were assessed during a 10-min period after releasing the occlusion and restoring blood flow. Any survivors were killed by exanguination.

Arrhythmias were monitored by continuous recording of electrocardiogram and blood pressure and identified as ventricular premature beats (VPB), ventricular tachycardia (VT), or ventricular fibrillation as reported earlier (3,5). The severity of arrhythmia was assessed by computing an arrhythmia score (A.S.) which takes into account both the frequency and duration of various parameters of arrhythmia (3,14). At the end of the experiment, the heart was removed and perfused with buffer, and the underperfused region was estimated by dye exclusion of Evans blue dye. The nonischemic and ischemic regions of the heart were quickly frozen in liquid N_2 and stored at –80°C for biochemical and analytical studies.

Measurement of cyclooxygenase metabolites. Frozen myocardial tissue was pulverized using a precooled (in liquid N_2) stainless-steel mortar and pestle. The powdered tissue was divided into several portions and suspended in ice-cold calcium-free Tyrodes medium (mM: 138 NaCl, 2.4 KCl, 3.1 Na_2HPO_4, 1.2 KH_2PO_4, 0.5 $MgCl_2$, pH 7.4). The samples were washed several times to remove any residual contamination with blood and incubated at 37°C for 45 min in this medium containing 1 mM Ca^{++} (9). Samples were rapidly cooled on ice and centrifuged at the end of the incubation period. Aliquots of the supernatant were analyzed for the stable metabolites of prostacyclin (PGI_2; measured as 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane A_2 (TxA_2; quantified as thromboxane B_2) by enzyme-immunoassay technique using kits purchased from the Caymen Chemical Co. (Ann Arbor, MI).

Measurement of lipoxygenase products. Powdered myocardial tissue was washed several times as described previously and finally incubated in a modified Tyrodes solution which contained, in addition to the aforementioned, L-cysteine (2 mmol/L) and increased Ca^{++} and Mg^{++} (2 mmol/L). After a 5-min preincubation period, the tissue suspensions were stimulated with ionophore, A23187 (0.5 μM), and incubated for a further 40 min. The reaction was terminated by cooling to 0°C and centrifugation. Supernatants were acidified (pH 3.5) with HCl before loading onto a C-18 Sep Pak (Waters-Millipore, Milford, MA) cartridge. The Sep Pak was washed with solutions of acetonitrile and water (25 and 20%, respectively) before the compounds of interests were eluted with acetonitrile. The solvent was removed under reduced pressure using a high-speed concentrator (Savant Speedvac SC100; Savant Instruments, Farmindale, NY), and samples were reconstituted in high-performance liquid chromatography-grade methanol. Recovery experiments using external standards showed a 65–70% efficiency of extraction. Dry weights of the tissue pellets were determined after extraction with 2 mL of acetone.

Lipoxygenase products were separated and quantified by high-performance liquid chromatography (Pharmacia-LKB, Bromma, Sweden) using a Brownlee (Applied Biosystems, Foster City, CA) Velosep C-18 reverse-phase analytical column (3.2 × 100 mm; 3 μm). The mobile phase consisted of 77.5% methanol and 0.1% trifluoroacetic acid with the flow rate maintained at 0.5 mL/min. Absorbance of the column eluent was continously monitored using two detectors in series, an LKB 2141 variable wavelength monitor set at 278 nm (for leukotrienes) and an LKB 2151 variable wavelength

monitor set at 235 nm for the detection of hydroxy fatty acids. Quantification was based upon peak areas relative to those of a standard mixture which contained prostaglandin B_2, leukotriene B_4, leukotriene C_4, and 5-, 12-, and 15-hydroxy-eicosatetraenoic acids. Data was collected and processed with the aid of a Nelson Analytical 920 interface and a Nelson 3000 series chromatography data system software package (PE Nelson, Cupertino, CA).

Statistics. The effects of different diets were compared against the SSO-supplemented diet, which was regarded as the reference point of the study. Data were analyzed using one-way analysis of variance followed by Tukey's test for multiple comparisons (15) between pairs at the 5% level or better.

RESULTS

Table 1 shows the major fatty acids of the experimental diets used in this study. It is clear that both the SSO and SBO diets are relatively rich in linoleic acid (43 and 59%, respectively) compared to the other three test diets, which contained low 18:2 levels (7–13%). Palm oil diets contained high proportions of palmitic (38%) and oleic (37%) acids, and no difference in fatty acid composition was evident between the NBDPO- and RBDPO-supplemented diets. The FO diet contained about 25% of total n-3 PUFA, with the two major fatty acids being eicosapentaenoic acid (EPA; 20:5n-3, 13%) and docosahexaenoic acid (22:6n-3, 9%). No *trans* fatty acid was detected in the SBO diet as the total *trans* fatty acid content of this SBO supplement was relatively small (<6%; data not shown).

All the diets were well accepted by the rats, as is evident from the consistent gains in body weights throughout the long-term (nine months) feeding period. The animals were healthy at the end of the pre-feeding period, and no obvious signs of any disease (e.g., yellow fat) were detected in major body organs at autopsy. The final body weights at the conclusion of the experiment were: SSO, 564.0 ± 8.5 g; SBO, 552.9 ± 7.4 g; NBDPO, 567.3 ± 5.9 g; RBDPO, 561.6 ± 7.1 g; and FO, 574.4 ± 8.1 g; n = 18 per group, $P > 0.05$.

TABLE 1
Major Fatty Acids[a] of Experimental Diets

	Diet[b]				
Fatty acid	SSO	SBO	NBDPO	RBDPO	FO
16:0	8.2	12.5	38.5	37.2	18.4
16:1	0.2	0.5	—	—	5.3
18:0	4.8	7.9	4.5	4.6	3.9
18:1n-9	22.0	25.2	36.3	37.0	14.6
18:2n-6	58.9	43.5	13.5	13.1	7.1
18:3n-3	0.9	2.8	0.8	0.7	1.4
20:1	—	—	—	—	4.3
20:5n-3	—	—	—	—	12.8
22:5n-3	—	—	—	—	1.8
22:6n-3	—	—	—	—	9.1

[a]Values are expressed as wt% and are the means of at least eight separate determinations.

[b]SSO, sunflower seed oil; SBO, partially hydrogenated soybean oil; NBDPO, neutralized, bleached, and deodorized palm oil; RBDPO, refined, bleached, and deodorized palm oil; and FO, fish oil.

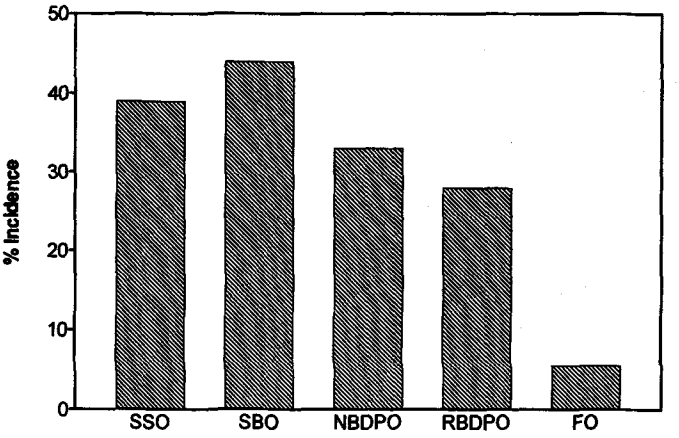


FIG. 1. Incidence (%) of ventricular tachycardia (VT) during experimentally-induced myocardial ischemia (5 min) in rats. Animals were fed different lipid supplemented diets for nine months before being subjected to regional myocardial ischemia and reperfusion as described in the text. SSO, sunflower seed oil; SBO, partially hydrogenated soybean oil; NBDPO, neutralized, bleached, and deodorized palm oil; RBDPO, refined, bleached, and deodorized palm oil; FO, fish oil; n = 18 per dietary group.

The effect of long-term feeding of different lipid supplements on various parameters of cardiac arrhythmia are presented in Figure 1 and in Table 2. As described in the Materials and Methods section, regional myocardial ischemia (5 min) was induced by ligating the left coronary artery followed by restoration of blood flow (reperfusion) for a 10-min period.

Figure 1 shows the % incidence of VT during ischemia where the protective effects of dietary n-3 PUFA can be seen. For example, in the reference group (SSO diet) 7 animals (7/18 or 39% incidence) displayed VT compared to only one animal (1/18) in the FO supplemented diet group. RBDPO and NBDPO groups showed 28 and 33% VT incidences, respectively, whereas in the SBO group 8/18 rats (44%) had developed tachycardia. However, these episodes of VT during this brief ischemic episode did not deteriorate into ventricular fibrillation in a majority of the animals, irrespective of the dietary group (data not shown).

The main parameters of cardiac arrhythmia following the restoration of blood flow (reperfusion) to the ischemic region are presented in Table 2. It is evident that only the NBDPO dietary group registered an increase in total VPB during the 10-min period compared to the SSO group ($P < 0.01$). As seen during the brief ischemic phase (Fig. 1), FO feeding resulted in the lowest incidence of VT among all groups. The mean duration of VT was also the lowest in the FO group, whereas NBDPO-fed rats displayed an increase relative to the linoleate rich SSO diet ($P < 0.05$ analysis of variance, Tukey's test; data not shown). Interestingly, the two palm oil preparations appeared to behave differently because, compared to the NBDPO group, RBDPO dietary rats showed a shorter duration of VT (26.8 s compared to 64.7 s for NBDPO). The A.S. were low in the SSO and FO groups compared to other three groups ($P < 0.05$ vs. SSO). No differences were evident in arrhythmic vulnerability between SBO and palm oil diet-fed animals.

TABLE 2
Effect of Various Lipid Supplements on Reperfusion-Induced (10 min) Arrhythmias[a]

Parameter[a]	Dietary group				
	SSO	SBO	NBDPO	RBDPO	FO
VPB/10 min	108 ± 45	298 ± 92	526 ± 111[b]	368 ± 96	94 ± 44
VT incidence (%)	7/18 (39%)	13/17 (76%)	12/17 (70%)	14/18 (77%)	4/18 (22%)
VF incidence (%)	2/18 (11%)	4/17 (23%)	6/17 (35%)	6/18 (33%)	1/18 (5%)
Total mortality[c]	1/18	2/18	1/18	2/18	0/18
A.S.	1.5 ± 0.5	3.1 ± 0.5[b]	3.2 ± 0.5[b]	3.3 ± 0.6[b]	0.9 ± 0.4

[a]For abbreviations Table 1; VPB, ventricular premature beat; VT, ventricular tachycardia; VF, ventricular fibrillation; A.S., arrhythmic score.
[b]Indicates significant difference at the 5% level (vs. SSO group, analysis of variance, Tukey's test).
[c]Including deaths for VF during the 5 min preceding ischemic period.

The major PUFA of cardiac membrane phospholipids which have been reported to affect eicosanoid production are presented in Table 3. Compared to the SSO diet, FO feeding led to an effective replacement of 18:2 (54% reduction), and palm oil diets showed a 26% reduction. However, despite this reduced level of 18:2 in palm oil-fed rats, the immediate elongation product of linoleic acid, i.e. arachidonic acid (AA 20:4n-6), was unaltered. Only the FO-fed animals demonstrated a significant reduction in this major fatty acid substrate for eicosanoids. The reduction in n-6 fatty acids in the myocardial phospholipids of FO-fed animals was offset by a considerable incorporation of n-3 PUFA (20:5, 22:5, and 22:6) which amounted to 24% of total fatty acids of myocardial membrane phospholipids. Nevertheless, the proportion of EPA in myocardial phospholipids remained low, being only 1.6% of total fatty acids, even after prolonged feeding of a diet rich (Table 1) in this long-chain PUFA.

Table 4 shows the release of PGI_2 and TxA_2 by homogenates of the ischemic/reperfused myocardium. The myocardial tissue from RBDPO-fed rats tended to have a greater capacity to produce PGI_2 following ischemia and reperfusion, than did the SSO group, although this difference did not achieve statistical significance. This possible increase in PGI_2 in the RBDPO group was not seen in the NBDPO group, despite both palm oil supplements containing an identical dietary fatty acid profile (Table 1). The production of TxA_2 was only affected by the n-3 PUFA-enriched diet. Therefore, the FO rats displayed a significant reduction in TxA_2 (51% reduction compared to the SSO group), which is not explained on the basis of reduction in membrane AA alone, which was approximately 15% lower in the FO group. These changes were reflected in the overall ratio of PGI_2/TxA_2, which was higher in both FO and RBDPO groups as compared to other dietary groups. There was no difference in PGI_2 production from the nonischemic region of the heart.

The production of lipoxygenase (LO) metabolites by cardiac muscle homogenates also was investigated in the present study. The major LO product of the heart was found to be 12-hydroxyeicosatetraenoic acid (12-HETE), which showed a significant response to dietary lipid supplementation (Fig. 2). The highest production of 12-HETE was associated with high linoleate diets (SSO 591.9 ± 95.8 ng/g dry wt; SBO, 375.5 ± 48.9 ng/g dry wt; $P > 0.05$), whereas both palm oil and FO feeding resulted in a lower production. As observed for PGI_2 production, homogenates from RBDPO-fed rats showed a tendency toward lower release of 12-HETE than did NBDPO. The % reductions relative to the SSO diet were: NBDPO (51%), RBDPO (61%), and FO (66%), but the differences between the two palm oil groups did not achieve statistical significance. In contrast to the AA-derived metabolites, no significant formation of LO products derived from EPA (e.g., hydroxyeicosapentaenoic acid) was observed possibly because of its low level of incorporation (only 1.6 vs. 17.3% for AA) into myocardial phospholipids. Similarly, the release of leukotriene

TABLE 3
Major Polyunsaturated Fatty Acids[a] of Myocardial Membrane Phospholipids[b]

Fatty acid	Dietary Groups[b]				
	SSO	SBO	NBDPO	RBDPO	FO
18:2n-6	14.9	14.8	11.1[c]	10.4[c]	6.8[c]
20:4n-6	21.4	20.5	19.6	21.0	17.3
20:5n-3	—	—	—	—	1.6[c]
22:5n-3	0.8	1.6[c]	1.1	1.4	2.0[c]
22:6n-3	10.3	13.7	14.1	14.6	20.5[c]
n-6/n-3 ratio	3.3	2.3	2.0	1.9	1.1

[a]Expressed as wt% of total phospholipid fatty acids.
[b]For abbreviations see Table 1.
[c]$P < 0.05$ (vs. SSO, analysis of variance, Tukey's test) values represent the means of n = 9 determinations.

TABLE 4
Effect of Various Lipid Supplements on the Release of PGI_2 and TxA_2 Following Myocardial Ischemia and Reperfusion[a]

Dietary group	ng/g tissue dry wt		
	PGI_2	TxA_2	PGI_2/TxA_2
SSO	430.1 ± 59.4	56.1 ± 6.8	7.7
SBO	354.0 ± 56.3	46.3 ± 6.2	7.6
NBDPO	382.6 ± 52.2	50.8 ± 6.9	7.5
RBDPO	562.8 ± 81.3	58.2 ± 7.9	9.7
FO	277.2 ± 35.5	27.8 ± 3.4[b]	10.0

[a]Prostaglandin I_2 (PGI_2) and thromboxane A_2 (TxA_2) were quantified as their stable end products 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2. Values refer to the mean ± SEM (n = 15–18). See Table 1 for abbreviations.
[b]$P < 0.05$ (vs. SSO, analysis of variance, Tukey's test).

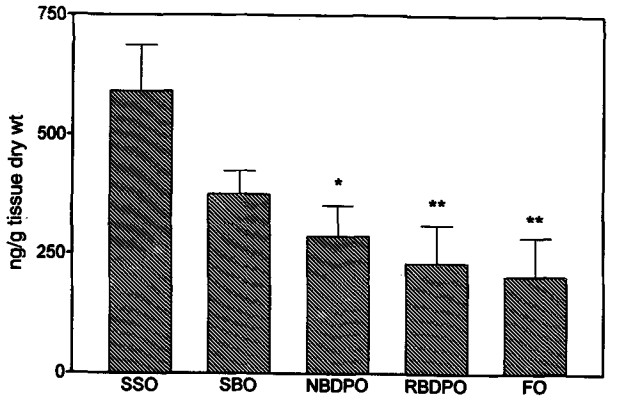


FIG. 2. Production of 12-hydroxyeicosatetraenoic acid by the ischemic/reperfused myocardium. Experimental conditions are described in detail in the Materials and Methods section. Values given are mean ± SEM; n = 15. Significant differences were assessed by analysis of variance followed by Tukey's post-hoc test for multiple comparisons (Ref. 15). $*P < 0.05$; $**P < 0.01$ vs. SSO. Abbreviations as in Figure 1.

B_4 or leukotrienes C_4/D_4 by the cardiac muscle was too low to be measured with accuracy using the assay methods employed in the present study.

Analysis of the vitamin E profile of different oil supplements showed SSO and palm oil preparations to be high in this antioxidant, whereas SBO contained no detectable level of α-tocopherol. Purified FO (MaxEPA®) preparation contained a high amount of α-tocopherol added by the manufacturer. The total vitamin E levels were: SSO, 557 ppm; SBO, not detected (<10 ppm); NBDPO, 637 ppm; RBDPO, 628 ppm; and FO, 958 ppm. Tocotrienols were only found in palm oils and amounted to about 75% of total tocopherols (data not shown).

DISCUSSION

The antiarrhythmic actions of dietary SSO and FO reported earlier (2,3) were confirmed in the present study. However, the effects of SBO, which contained a relatively high level of linoleic acid (18:2n-6; 43%), were indistinguishable from those of the diets rich in palm oils, which had only 13% of dietary linoleate. For example, the overall arrhythmic potential as expressed by the A.S. was not different between the SBO, NBDPO, and RBDPO groups. The two palm oil diets, however, appeared to exert differential effects on certain individual parameters of arrhythmia as well as on eicosanoid production. For example, both VPB and VT duration were greater following the NBDPO diet, despite this oil having a similar fatty acid profile to RBDPO. This suggests that the fatty acid profile of edible oils alone is not a predictor of arrhythmic vulnerability, and implies an important role for accompanying nonfatty acid constituents of dietary oils. The best candidates for such components are the endogenous antioxidants, as arrhythmogenesis has been reported to be influnced by both natural and synthetic antioxidants (16,17). In this context, it is worth noting that SBO used in the present study contained no α-tocopherol, whereas SSO and palm oils were high in this and other forms of vitamin E. Supporting evidence for the existence of a complex interaction between fatty acids and endogenous antioxidants in determining the overall arrhythmic vulnerability also arises from the recent observation that dietary olive oil, high in 18:1, failed to offer protection against arrhythmogenesis (5). Therefore, based on the fatty acid profile, palm oils with high 18:1 and low 18:2 levels could be expected to behave differently from linoleic acid-rich SBO. Nevertheless, no difference was evident in the relative arrhythmic potential of these dietary oils.

The fatty acid composition of cardiac membranes also tends to support this speculation because both SSO and SBO diets had similar effects on major PUFA (18:2; 20:4), but the two dietary treatments resulted in widely different physiological responses. Furthermore, and similar to our previous findings, the effects of dietary lipid supplements on the production of TxA_2 are also difficult to explain on the basis of precursor/inhibitor fatty acid availability alone (9,10,18). In the present study, FO feeding led to a considerably reduced capacity of the ischemic and reperfused myocardial tissue to produce TxA_2 and, in addition, limited the *in vitro* production of 12-HETE, a potent chemotactic and vasoactive agent which plays a pivotal role in the evolution of cardiac injury (19). Interestingly, this inhibitory action on 12-HETE production also was shared by palm oils.

The present findings also suggest that cardiac LO may have a broader substrate specificity in that it may be able to utilize both linoleic acid and AA as precursors for hydroxy fatty acids. Although EPA has been reported to be a preferred substrate for the LO enzyme complex (20), no end products of EPA metabolism were detected. This finding, however, can be explained on the basis of the availability of substrate fatty acid as the FO dietary animals showed negligible incorporation of EPA into the myocardial phospholipids. It appears that, in the rat myocardium, EPA is either poorly incorporated or further elongated and desaturated to yield docosahexaenoic acid (Tables 1,3).

The greater efficacy of RBDPO compared to NBDPO to limit the release of 12-HETE also suggests a modulatory role for minor constituents of dietary oils. However, tocopherols can be ruled out as a potential candidate, as both RBDPO and NBDPO contained comparable levels of this antioxidant. Trace metals (particularly selenium) offer an attractive explanation as recent reports indicate a potent modulatory role for selenium on the metabolism of eicosanoids (21). Another possible explanation, however, lies with the presence or absence of nontocopherol-type antioxidants in the two types of palm oil used in the present study. For example, several different polyphenol compounds (e.g., bioflavanoids) of plant origin have been shown to exert potent antioxidant actions and to modulate eicosanoid biosynthesis (22,23). In this regard, it is of interest to point out that increased aortic production of PGI_2 following olive oil feeding, compared to a high-oleic safflower oil diet, is reported to be mediated *via* polyphenol constituents of olive oil (24), which are known to be influenced by the refining process (25). Polyphenol antiox-

idants could lower the fatty acid hydroperoxide levels by inhibiting LO enzyme complex, thereby increasing PGI_2 synthesis through removal of the inhibitory effects of fatty acid hydroperoxide on prostacyclin synthetase (26). In the present study, RBDPO was found to exert trends toward both these effects—inhibition of 12-HETE formation and increase in PGI_2 synthesis. Therefore, it is conceivable that differences in the refining method (i.e., physical, RBDPO vs. chemical, NBDPO) may result in some biological differences between the two preparations of palm oils. Further studies are clearly warranted in this area.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Palm Oil Research Institute of Malaysia (PORIM). We thank A. Gapor (PORIM) for tocopherol analysis.

REFERENCES

1. Schaper, A.G. (1988) *Coronary Heart Disease Risks and Reasons*, pp. 1–41, Current Medical Literature Ltd., London.
2. Charnock, J.S. (1994) Lipids and Cardiac Arrhythmia, *Prog. Lipid. Res. 33*, 355–401.
3. McLennan, P.L., Abeywardena, M.Y., and Charnock, J.S. (1988) Dietary Fish Oil Prevents Ventricular Fibrillation Following Coronary Artery Occlusion and Reperfusion, *Am. Heart. J. 16*, 709–717.
4. McLennan, P.L., Bridle, T.M., Abeywardena, M.Y., and Charnock, J.S. (1992). Dietary Lipid Modulation of Ventricular Fibrillation Threshold in the Marmoset Monkey, *Am. Heart J. 123*, 1555–1561.
5. McLennan, P.L. (1993) Relative Effects of Dietary Saturated, Monounsaturated and Polyunsaturated Fatty Acids on Cardiac Arrhythmias in Rats, *Am. J. Clin. Nutr. 57*, 207–212.
6. Manning, A.S., and Hearse, D.J. (1984) Reperfusion Induced Arrhythmias: Mechanisms and Prevention, *J. Molec. Cell. Cardiol. 16*, 497–518.
7. Parratt, J. (1993) Endogenous Myocardial Protective (antiarrhythmic) Substances, *Cardiovasc. Res. 27*, 693–702.
8. Lefer, A. (1985) Eicosanoids as Mediators of Ischemia and Shock, *Federation Proc. 44*, 275–280.
9. Abeywardena, M.Y., McLennan, P.L., and Charnock, J.S. (1991) Differential Effects of Dietary Fish Oil on Myocardial Prostaglandin I_2 and Thromboxane A_2 Production, *Am. J. Physiol. 260*, H379–385.
10. Abeywardena, M.Y., McLennan, P.L., and Charnock J.S. (1993) Role of Eicosanoids in Dietary Fat Modification of Cardiac Arrhythmia and Ventricular Fibrillation, in *Essential Fatty Acids and Eicosanoids* (Sinclair, A., and Gibson, R., eds.), pp. 257–261, AOCS Press, Champaign.
11. Charnock, J.S., McLennan, P.L., Abeywardena, M.Y., and Russell, G.R. (1985) Altered Levels of n-6/n-3 Fatty Acids in Rat Heart and Storage Fat Following Variable Dietary Intake of Linoleic Acid, *Ann. Nutr. Metab. 29*, 279–288.
12. Charnock, J.S., Abeywardena, M.Y., and McLennan, P.L. (1986) Comparative Changes in the Fatty Acid Composition of Rat Cardiac Phospholipids After Long-Term Feeding of Sunflower Seed Oil or Tuna Fish Oil Supplemented Diets, *Ann. Nutr. Metab. 30*, 393–406.
13. Charnock, J.S., Abeywardena, M.Y., McMurchie, E.J., and Russell, G.R. (1984) The Composition of Cardiac Phospholipids in Rats Fed Different Lipid Supplements, *Lipids 19*, 206–213.
14. Curtis, M.J., and Walker, M.J.A. (1988) Quantification of Arrhythmias Using Scoring Systems: An Examination of Seven Scores in an *in vivo* Model of Regional Myocardial Ischemia, *Cardiovasc. Res. 22*, 656–665.
15. Wallenstein, S., Zucker, C.L., and Fleiss, J.L. (1980) Some Statistical Methods Useful in Circulation Research, *Circ. Res. 47*, 1–9.
16. Simpson, P.J., Mickelson, J.K., and Lucchesi, B.R. (1987) Free Radical Scavengers in Myocardial Ischemia, *Federation Proc. 46*, 2413–2421.
17. Fuenmayor, A.J., Fuenmayor, A.M., Lopez, T., and Winterdaal, D.M. (1989) Vitamin E and Ventricular Fibrillation Threshold in Myocardial Ischemia, *Jpn. Circ. J. 53*, 1229–1232.
18. Abeywardena, M.Y., McLennan, P.L., and Charnock, J.S. (1991) Differences Between *in vivo* and *in vitro* Production of Eicosanoids Following Long-Term Dietary Fish Oil Supplementation in the Rat, *Prost. Leuk. Essential Fatty Acids 42*, 159–165.
19. Kuzuya, T., Hoshida, S., Nishida, M., Kim, Y., Kamada, T., and Tada, M. (1987) Increased Production of Arachidonate Metabolites in Occlusion-Reperfusion Model of Canine Myocardial Infarction, *Cardiovasc. Res. 21*, 557–568.
20. Terano, T., Salmon, J.A., and Moncada, S. (1984) Effect of Orally Administered Eicosapentaenoic Acid (EPA) on the Formation of Leukotriene B_4 and Leukotriene B_5 by Rat Leukocytes, *Biochem. Pharmacol. 33*, 3071–3076.
21. Raddano, P., Whelan, J., Burgess, J.R., Eskew, M.L., Hildenbrandt, G., Zarkower, A., Scholz, R.W., and Reddy, C.C. (1989) The Role of Vitamin E and Selenium on Arachidonic Acid Oxidation by Way of the 5-Lipoxygenase Pathway, *Ann. NY. Acad. Sci. 570*, 136–145
22. Gryglewski, R.J., Korbut, R., Robak, J., and Swies, J. (1987) On the Mechanisms of Antithrombotic Action of Flavanoids, *Biochem. Pharmacol. 36*, 317–322.
23. Janero, D.R., Burghardt, B., Lopez, R. and Cardell, M. (1989) Influence of Cardioprotective Cyclooxygenase and Lipoxygenase Inhibitors on Peroxidative Injury to Myocardial Membrane Phospholipid, *Biochem. Pharmacol. 38*, 4381–4387.
24. Blakenship, J.W., Jenks, R., MacMurray, A.M., Sandberge, L.B., and Boucek, R.J. (1989) Uniqueness of Dietary Olive Oil in Stimulating Aortic Prostacyclin Production in Post-Weanling Rats, *Prost. Leuk. Essential Fatty Acids 36*, 31–34.
25. Gutfinger, T. (1981) Polyphenols in Olive Oils, *J. Am. Oil. Chem. Soc. 58*, 966–968.
26. Gisinger, C., Watanabe, J., and Colwell, J.A. (1990) Vitamin E and Platelet Eicosanoids in Diabetes Mellitus, *Prost. Leuk. Essential Fatty Acids 40*, 169–176.

[Received November 19, 1993; and in final revised form September 5, 1995; Revision accepted September 27, 1995]

Plasma Lipids Are Affected Similarly by Dietary Lauric or Palmitic Acid in Gerbils and Monkeys[1]

Andrzej Pronczuk, Pramod Khosla, Tahar Hajri, and K.C. Hayes*

Foster Biomedical Research Laboratory, Brandeis University, Waltham, Massachusetts 02254

ABSTRACT: To compare the relative impact of dietary lauric acid (12:0) and palmitic acid (16:0) on plasma lipids, two fat-sensitive species, Mongolian gerbils and cebus monkeys, were fed cholesterol-free, purified diets enriched with either 12:0-rich or 16:0-rich fats, while all other fatty acids were held constant by selective blending of up to five natural fats or oils. The two gerbil diets (40 en% from fat) allowed for an 8 en% exchange between 12:0 and 16:0, and the monkey diets (31 en% from fat) allowed for 6 en% exchange between these two fatty acids. Eight gerbils received the diets for eight weeks, and 12 cebus monkeys were fed each diet in a cross-over design for up to 22 wk. Both diets resulted in similar plasma cholesterol, triglyceride, and high density lipoprotein cholesterol concentrations within each species. Additionally, separation of cebus lipoproteins by discontinuous density-gradient ultracentrifugation failed to show any dietary differences in concentration or composition of the three major lipoprotein classes (d < 1.019, 1.019–1.055, and 1.055–1.168 g/mL). Thus, in two species sensitive to manipulations in dietary fat while consuming cholesterol-free diets, 16:0 was not hypercholesterolemic relative to 12:0.

Lipids 30, 1157–1161 (1995).

The ability of dietary saturated fat to raise plasma cholesterol has been appreciated for almost four decades (see Refs. 1–4 for recent reviews). More than thirty years ago, Keys *et al.* (5) proposed that the saturated fatty acids (12:0, 14:0, 16:0) were equally capable of raising cholesterol, while the saturated stearic acid (18:0) and monounsaturated oleic acid (18:1) were neutral. Hegsted *et al.* (6) concurred, in general, but initially found 14:0 to be substantially more cholesterolemic than either 12:0 or 16:0. However, subsequent experiments with synthetic triglycerides (7) led the latter investigators to the same conclusion as Keys *et al.* (5), although they included the small intake of 18:0 among the "saturates" pool. Numerous studies in the last decade have challenged the earlier notion that the C_{12}–C_{16} saturated fatty acids were equivalent with respect to their effects on serum cholesterol.

Although there is general agreement on the neutrality of 18:0 (7–11), considerable disagreement exists on the roles of lauric (12:0), myristic (14:0), and palmitic (16:0) acids in modulating plasma lipids. Studies in hamsters fed trilaurin-, trimyristin-, or tripalmitin-enriched diets revealed that all three (12:0, 14:0, and 16:0) were equivalent in terms of their cholesterol-raising ability (12). Denke and Grundy (13) reported that 16:0 was hypercholesterolemic relative to 12:0 in human subjects consuming liquid-formula diets containing a modified triglyceride enriched in 12:0, and both Doherty and Iacano (14) and Schwab *et al.* (15) observed similar cholesterol values when humans consumed natural fats and whole-food diets in which 12:0 was exchanged for 16:0.

Recent studies from this laboratory using normocholesterolemic hamsters (16), cebus and rhesus monkeys (17), and gerbils (18) consuming cholesterol-free diets, as well as collaborative studies in normocholesterolemic human subjects consuming whole-food diets providing about 200 mg cholesterol per day (19,20), suggest that if low density lipoprotein (LDL) receptor activity is not compromised, diets rich in 12:0 + 14:0 will increase plasma and LDL cholesterol, and 16:0 (similar to 18:1) can be quite neutral in its impact on plasma cholesterol. The latter interpretation is consistent with the initial "best" multiple regression equation of Hegsted *et al.* (6) that indicated 14:0 was four times more potent than 16:0 in its ability to raise the plasma cholesterol in men consuming whole-food diets containing natural fats. This contrasts with the situation in which LDL receptor activity would be impaired, as in hypercholesterolemic humans (21) or non-human primates fed dietary cholesterol (22,23), where 16:0 has been found to elevate plasma cholesterol. Re-analysis (24) of the original data reported by Hegsted *et al.* (6) confirmed this premise. In subjects consuming <300 mg/d cholesterol, 16:0 appeared neutral, whereas a hypercholesterolemic effect of 16:0 was observed in subjects consuming >400 mg/d cholesterol (24). More recently, Fielding *et al.* (25) have confirmed the dependence of "the saturated fat effect" on the presence of dietary cholesterol in normocholesterolemic subjects, including the possibility that ethnic background or lifestyle can influence the outcome. Furthermore, multiple regression analysis of accumulated data from normocholesterolemic cebus monkeys (24) and gerbils (18) implied that dietary 14:0 and 18:2 were the only two fatty acids to impact the plasma cholesterol concentration when LDL receptor activity was not impaired, i.e., when normal plasma cholesterol values exist at the time of intervention.

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, September 1993.

*To whom correspondence should be addressed.

Abbreviations: HDL, high density lipoprotein, LDL, low density lipoprotein.

Copyright © 1995 by AOCS Press

The current study was designed to address the question whether 12:0 *per se* contributes to the hypercholesterolemia previously observed during feeding of 12:0 + 14:0-rich diets to normocholesterolemic animals. Based on our regression data, indicating that only dietary 14:0 (cholesterol-raising) and 18:2 (cholesterol-lowering) induce measurable changes in plasma cholesterol, we reasoned that in the absence of cholesterol, dietary 12:0, like 16:0, should be neutral and their direct exchange should not perturb plasma lipids. The results agree with our prediction and further the notion originally proffered by Hegsted *et al.* (6) that the exceptional hypercholesterolemia observed by feeding 12:0 + 14:0-rich diets depends, in part, upon the presence of 14:0.

MATERIALS AND METHODS

Animals, diets, and study design. Twelve male cebus monkeys (*Cebus albifrons*)10–20 years old, and 16 male, eight-month-old Mongolian gerbils (*Meriones unguiculatus*; Tumblebrook Farm, West Brookfield, MA) were used in this study. The cebus monkeys had been fed purified diets from birth, and immediately prior to this investigation had been fed for eight weeks with a low-fat (20 en%), cholesterol-free diet containing equal amounts of saturated, monounsaturated, and polyunsaturated fatty acids. Mean (±SEM) plasma lipid values for the 12 monkeys at entry were: total cholesterol, 142 ± 10 mg/dL; and triglycerides, 89 ± 20 mg/dL.

The basal composition of the purified monkey diets was (g/kg dry weight): lactalbumin, 190; cornstarch, 256; sucrose, 255; cellulose, 100; fat, 143; mineral mix, 48; vitamin mix, 5; and choline chloride, 3. The composition of the Ausman–Hayes mineral and Newberne–Hayes vitamin mixes has been reported (17). For the 16:0-rich diet, the fat blend comprised (g/100 g fat): 55 g cholesterol-stripped milkfat, 20 g palm stearin, 15 g coconut oil, 5 g safflower oil, and 5 g olive oil. The 12:0-rich fat blend contained 55 g coconut oil and 45 g olive oil. The purified gerbil diets consisted of (g/kg dry weight): casein, 222; cornstarch, 230; glucose, 133; cellulose, 150; fat, 200 (same blends as the monkey diets); Ausman–Hayes mineral mix, 50; Hayes–Cathcart vitamin mix, 12; and choline chloride, 3. The fat represented approximately 31 en% in the monkey diet, and 41 en% in the gerbil diet.

Monkeys were housed individually, and gerbils were housed in groups of 3–4 per cage from four weeks of age. All animals were kept in a controlled environment with a 12-h light/dark cycle and had free access to water. Food was provided daily in a predetermined ration calculated to maintain constant body weight for the duration of the study. All protocols and procedures were approved by the Brandeis University Animal Care and Use Committee.

Animals were randomly assigned to either diet which differed solely in their source of fats and their fatty acid composition (Table 1), the latter verified by gas–liquid chromatography (26). Gerbils were fed their diets for eight weeks (eight gerbils/diet). For the monkeys a random cross-over design was employed, i.e., initially (phase 1) half of the cebus (n = 6) were assigned to each diet for an eight-week period. At the end of this period, diets were switched for another eight weeks. To ascertain any potential long-term effects of the diets on plasma lipids, feeding was continued for an additional 14 wk (phase 2: 22 wk total). Plasma lipid analyses were carried out at the end of eight (n = 12) weeks for the gerbils, whereas analyses in monkeys were carried out at 8 (n = 12) and 22 wk (n = 6).

TABLE 1
Fatty Acid Composition of Purified Diets as Fed to Cebus Monkeys and Gerbils[a]

	Percentage of total fatty acids	
Fatty acid	16:0-rich diet	12:0-rich diet
<12:0	5.6	6.6
12:0	12.2	32.9
14:0	10.3	9.7
16:0	35.9	11.2
18:0	5.7	1.2
18:1	22.3	31.4
18:2	6.2	6.2
18:3	0.1	0.1
Total sats	69.7	61.6
Total monos	22.3	31.4
Total polys	6.2	6.2
P/S	0.09	0.10

[a]Fatty acid profile represents a blend of fats and was generated by gas–liquid chromatographic analysis (see the Materials and Methods section). Sats, saturated fatty acids; monos, monounsaturated fatty acids; polys, polyunsaturated fatty acids; P/S, polyunsaturated/saturated fatty acid ratio.

Plasma lipid and lipoprotein determinations. Following an overnight fast (14–16 h), monkeys were sedated with an intramuscular injection of Ketamine HCl (10 mg/100 μL, Vetalar; Parke-Davis, Morris Plains, NJ) at a dose of 4–9 mg/kg body weight. Blood was obtained from the femoral (or saphenous) vein and placed into tubes containing an EDTA solution kept on ice. Blood samples (500 μL) were procured from gerbils by cardiac puncture following light anesthesia ($CO_2 + O_2$, 50:50 mixture) using a 30# needle and an EDTA-wetted insulin syringe. Plasma was isolated by centrifugation. Whole plasma and individual lipoprotein fractions were assayed enzymatically for their cholesterol and triglyceride content (kits Nos. 352 and 336, respectively; Sigma Diagnostics, St. Louis, MO). High density lipoprotein (HDL) cholesterol concentrations were determined in the supernatant of plasma samples following precipitation with phosphotungstic acid/Mg^{2+} (for monkeys: HDL cholesterol reagent, No. 352-4, Sigma Diagnostics; and for gerbils: HDL cholesterol Reagent Set, Boehringer Diagnostics, Indianapolis, IN). Lipoprotein phospholipids were determined using the Phospholipid B enzymatic kit (Wako Chemicals, Richmond, VA). Lipoprotein protein was determined using the Markwell modification of the procedure of Lowry (27).

Isolation of cebus lipoproteins. At the end of the 22-wk period, cebus plasma (from individual animals) was subjected to discontinuous density-gradient ultracentrifugation essentially as described by Chapman *et al.* (28), with the subsequent modification introduced by Goulinet and Chapman

(29). Gradients were prepared in Beckman Ultra-Clear tubes (Beckman Instruments, Palo Alto, CA) by succesively layering 2 mL of d = 1.24 g/mL solution, 3 mL plasma @ d = 1.21 g/mL, 2 mL of d = 1.063 g/mL solution, 2.5 mL d = 1.019 g/mL solution, and 2.5 mL of d = 1.006 g/mL solution. Tubes were spun at 35,000 rpm (max acceleration but no brake) for 48 h at 15°C in a Beckman SW 41 rotor using a Beckman L6-60 optima ultracentrifuge.

Immediately following centrifugation, lipoproteins were collected in thirty fractions using a Gilson precision pipette (Rainin Instruments Co., Woburn, MA), starting with a top fraction of 500 µL and subsequent 400 µL fractions. The cholesterol, triglyceride, phospholipid, and protein content of each fraction was determined. A blank salt gradient also was ultracentrifuged and identical fractions collected for measurement of densities using a µOsmette micro osmometer (Precision Systems Inc., Sudbury, MA).

Statistical analyses. All statistical analyses were performed using a Macintosh LC III computer (Apple Computer Inc., Cupertino, CA) with the Statview 512$^+$ (Brain Power Inc., Calabasca, CA) and Cricket Graph (Cricket Software Inc., Philadelphia, PA) statistical packages. The effects of the dietary fatty acids were assessed using Student's unpaired *t*-test (gerbils) or Student's paired *t*-test (monkeys). In all cases, statistical significance was set at $P < 0.05$.

RESULTS

After eight weeks of diets enriched with 12:0 or 16:0, gerbil and cebus body weights averaged 94 ± 2 g and 3.2 ± 0.06 kg, respectively. No significant difference was detected between dietary groups. Similarly, neither gerbils nor cebus monkeys exhibited significant dietary differences in their plasma cholesterol, triglycerides, or HDL-cholesterol concentration (Table 2), even though all total cholesterol values were elevated relative to typical basal values for these species (75 mg/dL for gerbils and 145 mg/dL for cebus). Extending the diet period to 22 wk in cebus monkeys still failed to elicit significant differences in plasma lipids between dietary fatty acid groups, although cholesterol values for both diets tended to be higher after 22 wk than at 8 wk, and there was a nonsignificant trend toward lower values in the 16:0 diet than in the 12:0 at 22 wk. As only six monkeys were extended to 22 wk on each diet, statistical analysis was not deemed appropriate between 8 and 22 wk.

TABLE 2
Plasma Lipids Concentration in Gerbils and Cebus Monkeys Fed Purified 12:0- or 16:0-Rich Diets for 8 or 22 Weeks[a]

Lipids[b] (mg/dL)	Diets			
	Gerbil		Cebus	
	12:0-Rich	16:0-Rich	12:0-Rich	16:0-Rich
Eight weeks				
TC	111 ± 10	106 ± 11	192 ± 10	200 ± 10
HDL-C[c]	81 ± 8	71 ± 6	116 ± 10	128 ± 8
TG	153 ± 38	163 ± 14	57 ± 3	78 ± 10
Twenty-two weeks				
TC	ND[d]	ND	227 ± 11	211 ± 16
HDL-C[c]	ND	ND	127 ± 11	133 ± 11
TG	ND	ND	76 ± 9	67 ± 7

[a]Values are mean ± SEM. Eight gerbils were fed each diet. For monkeys, the eight-week values are for all 12 animals fed each diet (mean of both diet rotations), while the 22-wk value is the mean from six animals per diet (values from the second rotation only).
[b]TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides.
[c]HDL obtained by phosphotungstic acid–Mg precipitation (see the Materials and Methods section).
[d]ND, not determined.

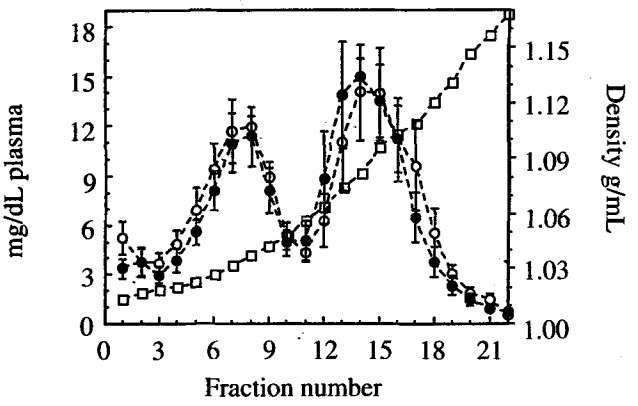


FIG. 1. Cholesterol and density distribution in cebus lipoproteins following density-gradient ultracentrifugation. The figure shows the cholesterol values (mean ± SEM) of six monkeys fed the 12:0-rich diet (○– – –○) and five monkeys (one sample of the 16:0-rich group was lost) fed the 16:0-rich diet (●– – –●). A blank salt gradient was used for measurment of densities (□– – –□).

Because similar plasma lipid concentrations do not necessarily mean that the lipoprotein distribution is the same, the cebus monkey lipoprotein classes were assessed across the entire density range after consuming either the 12:0-rich or 16:0-rich diets for 22 wk. Cebus lipoprotein cholesterol was distributed among three peaks with density less than 1.019, 1.019–1.055, and 1.055–1.168 g/mL (Fig. 1). The quantitative distribution and percent composition of lipoproteins in the three density classes were calculated with the result that dietary fatty acid treatment had no significant effect on any lipoprotein fraction (Table 3).

DISCUSSION

Our finding that dietary 12:0 and 16:0 exerted equivalent effects on plasma lipids is in agreement with previous reports in human subjects consuming whole-food diets (14,15), but disagrees with the conclusion of a study in humans consuming liquid-formula diets that found 16:0 to be more hypercholesterolemic than 12:0 (13). However, the latter study, as well as results from studies with liquid-formula diets in general, have been criticized because they may not reflect data obtained with solid-food diets (30). In addition, the 12:0 vs. 16:0 liquid formula comparison in humans (13) utilized modified triglyceride (monoacid) to incorporate a high level of 12:0 and was further biased by failure to equalize 14:0 and 18:2

TABLE 3
Effect of Dietary 12:0 and 16:0 for 22 Weeks on Cebus Lipoprotein Lipids and Protein Concentration and Lipoprotein Composition[a]

	Cholesterol (mg/dL plasma)	Triglyceride (mg/dL plasma)	Phospholipid (mg/dL plasma)	Protein (mg/dL plasma)
d < 1.019 g/mL density (fractions 1–3)				
12:0-Rich diet	15 ± 3	29 ± 8	15 ± 3	16 ± 11
	(22 ± 2)[b]	(37 ± 3)	(20 ± 1)	(21 ± 4)
16:0-Rich diet	12 ± 2	27 ± 9	13 ± 3	17 ± 6
	(17 ± 2)	(34 ± 7)	(18 ± 2)	(31 ± 8)
1.019 < d < 1.055 g/mL density (fractions 4–11)				
12:0-Rich	76 ± 7	25 ± 7	61 ± 7	84 ± 9
	(31 ± 1)	(10 ± 1)	(25 ± 1)	(35 ± 2)
16:0-Rich	68 ± 8	22 ± 3	57 ± 8	87 ± 11
	(29 ± 1)	(10 ± 1)	(24 ± 2)	(37 ± 3)
1.055 < d < 1.168 g/mL density (fractions 12–22)				
12:0-Rich	107 ± 6	35 ± 6	159 ± 17	324 ± 24
	(17 ± 1)	(5 ± 1)	(26 ± 2)	(52 ± 2)
16:0-Rich	102 ± 11	33 ± 2	182 ± 12	304 ± 35
	(16 ± 1)	(5 ± 0.5)	(30 ± 2)	(49 ± 3)

[a]Lipoproteins were isolated from the plasma of individual animals by discontinuous density gradient ultracentrifugation (see the Materials and Methods section). Values are the mean ± SEM for six monkeys fed the 12:0-rich diet and five monkeys fed the 16:0-rich diet (one sample of the 16:0-rich group was lost). No statistically significant differences were detected between dietary groups for any of the measured parameters.
[b]Values in parentheses denote % composition.

between diets (31,32). In humans fed transesterified triglycerides (33) or monoacid-enriched triglycerides (7), and in hamsters fed diets containing synthetic (monoacid) triglycerides and 0.12% (w/w) cholesterol (12), 12:0, 14:0, and 16:0 appeared to exert equal effects on plasma cholesterol. However, the hamster study cannot be compared directly with our results because 0.12% dietary cholesterol may have down-regulated LDL receptors sufficiently to cause all saturated fatty acids to appear to be equally cholesterol-elevating. In fact, in all three of these studies, the use of synthetic triglycerides may have distorted lipid metabolism and generated profiles for fatty acids that may not be easily extrapolated to the situation where those fatty acids are consumed as natural fats and oils (34,35).

The results of the current study also are consistent with a retrospective analysis of data from cebus monkeys fed numerous cholesterol-free diets (24), which was coupled with a reappraisal of the published data of Hegsted *et al.* (6). Both multiple regression analyses revealed that in normocholesterolemic subjects (humans or monkeys) consuming low-cholesterol diets, dietary 14:0 was the principal fatty acid associated with elevated plasma cholesterol. This observation was in accord with the original report of Hegsted *et al.* (6), in which 14:0 was assigned a fourfold higher cholesterol-elevating power than 16:0 in the "best" regression equation. In neither the Hegsted study (6) nor the monkey study (24) did multiple regression analysis identify a cholesterol-elevating role for 12:0 independent of 14:0. In a subsequent study with gerbils (18), incremental increases in dietary cholesterol confirmed the unique ability of a 16:0-rich fat to elicit progressive levels of cholesterolemia with increasing cholesterol intake. Thus, the cholesterol-raising potential of 16:0-rich fats appears to depend on the lipoprotein "setpoint" of the host at the time of challenge. As discussed elsewhere (35,36), the "setpoint" is highly dependent upon cholesterol intake and the 18:2 content of the diet, two factors greatly affecting LDL receptor activity (37).

In previous studies, diets rich in 12:0 + 14:0 were found to be hypercholesterolemic relative to 16:0-rich diets when formulated as part of cholesterol-free diets in gerbils (18) or three species of monkeys (17). The same result was obtained in normocholesterolemic human subjects consuming low-cholesterol (200 mg/d) diets (20). In order to ascertain the relative cholesterolemic impact of 12:0, the current diet exchanged 12:0 with 16:0 in the absence of cholesterol while maintaining a constant level of 14:0 and 18:2. Out of necessity to include adequate 12:0 while holding 14:0 constant in these two natural fat blends, substantial 14:0 (3–4 en%) was included as these two fatty acids normally occur together. Not surprisingly, the total cholesterol was elevated above basal values in both gerbils and cebus. However, relative to higher and lower consumption of 14:0-rich fat under similar circumstances, the present total cholesterol values were predictably intermediate to those previously obtained (17,18,24). Thus, the fact that 12:0 and 16:0 appeared similarly cholesterolemic implies that the more pronounced cholesterol elevation observed previously during feeding of 12:0 + 14:0-rich diets in comparison to 16:0-rich diets (6,17,20) may be attributed to the presence of 14:0 in those diets.

Coupled with our previous data (16,17,23,24,38–40), the current results suggest that 12:0 *per se* does not exert an independent cholesterol-elevating effect on plasma cholesterol in diets devoid of cholesterol [or in the case of human studies, diets with cholesterol intakes of <300 mg/d (24). In such situations, the presence of myristic acid (14:0), despite its low intake, would appear to dominate the cholesterolemic impact of saturated fatty acids on plasma cholesterol.

ACKNOWLEDGMENTS

This study was supported in part by the Palm Oil Research Institute of Malaysia, NIH grant HL49128, and by the Malaysian Palm Oil Development Council.

REFERENCES

1. *Diet and Health: Implications for Reducing Chronic Disease Risk*, Committee on Diet and Health, Food and Nutrition Board, Commission on Life Sciences, National Research Council (1989), pp. 159–258, National Academy Press, Washington, D.C.
2. Grundy, S.M. (1989) Monounsaturated Fatty Acids and Cholesterol Metabolism: Implications for Dietary Recommendations, *J. Nutr. 119*, 529–534.
3. Grundy, S.M., and Denke, M.A. (1990) Dietary Influences on Serum Lipids and Lipoproteins, *J. Lipid Res. 31*, 1149–1172.
4. Gurr, M.I. (1992) Dietary Lipids and Coronary Heart Disease: Old Evidence, New Perspective, *Prog. Lipid Res. 31*, 195–244.
5. Keys, A., Anderson, J.T., and Grande F. (1965) Serum Choles-

terol Response to Changes in the Diet. IV. Particular Saturated Fatty Acids in the Diet, *Metabolism 14*, 776–787.
6. Hegsted, D.M., McGandy, R.B., Myers, M.L., and Stare, F.J. (1965) Quantitative Effects of Dietary Fat on Serum Cholesterol in Man, *Am. J. Clin. Nutr. 17*, 281–295.
7. McGandy, R.B., Hegsted, D.M., and Meyers, M.L. (1970) Use of Semisynthetic Fats in Determining the Effects of Specific Dietary Fatty Acids on Serum Lipids in Man, *Am. J. Clin. Nutr. 23*, 1288–1298.
8. Grundy, S.M. (1994) Influence of Stearic Acid on Cholesterol Metabolism Relative to Other Long-Chain Fatty Acids, *Am. J. Clin. Nutr. 60*, 986S–990S.
9. Reiser, R., Probstfield, J.L., Silvers, A., Scott, L.W., Shorney, M.L., Wood, R.D., O'Brien, B.C., Gotto, A.M. Jr., and Insull, W. Jr. (1985) Plasma Lipid and Lipoprotein Response of Humans to Beef Fat, Coconut Oil and Safflower Oil, *Am. J. Clin. Nutr. 42*, 190–197.
10. Bonanome, A., and Grundy, S.M. (1988) Effect of Dietary Stearic Acid on Plasma Cholesterol and Lipoprotein Level, *N. Engl. J. Med. 318*, 1244–1248.
11. Kris-Etherton, P.M., Derr, J., Mitchell, D.C., Mustad, V.A., Russell, M.E., McDonnell, E.T., Salabsky, D., and Pearson, T.A. (1993) The Role of Fatty Acid Saturation on Plasma Lipids, Lipoproteins, and Apoproteins. I. Effects of Whole Food Diets High in Cocoa Butter, Olive Oil, Soybean Oil, Dairy Butter and Milk Chocolate on Plasma Lipids of Young Men, *Metabolism 42*, 121–129.
12. Woolett, L.A., Spady, D.K., and Dietschy, J.M. (1992) Saturated and Unsaturated Fatty Acids Independently Regulate LDL Receptor Activity and Production Rate, *J. Clin. Invest. 89*, 1133–1141.
13. Denke, M.A., and Grundy, S.M. (1992) Comparison of Effects of Lauric Acid and Palmitic Acid on Plasma Lipids and Lipoproteins, *Am. J. Clin. Nutr. 56*, 895–898.
14. Doherty, R.M., and Iacano, J.M. (1992) Dietary Lauric Acid Has Similar Effects on Blood Cholesterol as Palmitic Acid in Natural Diets in Man, *FASEB J. 6*, A1680.
15. Schwab, U.S., Niskanen, L.K., Maliranta, H.M., Savolainen, M.J., Kesaniemi, Y.A., and Uusiyupa, M.J. (1995) Lauric and Palmitic Acid-Enriched Diets Have Minimal Impact on Serum Lipid and Lipoprotein Concentrations and Glucose Metabolism in Healthy Young Women, *J. Nutr. 125*, 466–473.
16. Lindsey, S., Bennattar, J., Pronczuk, A., and Hayes, K.C. (1990) Dietary Palmitic Acid (16:0) Enhances HDL Cholesterol and LDL Receptor mRNA in Hamsters, *Proc. Soc. Exp. Biol. Med. 195*, 261–269.
17. Hayes, K.C., Pronczuk, A., Lindsey, S., and Diersen-Schade, D. (1991) Dietary Saturated Fatty Acids (12:0, 14:0, 16:0) Differ in Their Impact on Plasma Cholesterol and Lipoproteins, *Am. J. Clin. Nutr. 53*, 491–498.
18. Pronczuk, A., Khosla, P., and Hayes, K.C. (1994) Dietary Myristic, Palmitic, and Linoleic Acids Modulate Cholesterolemia in Gerbils, *FASEB J. 8*, 1191–1200.
19. Ng, T.K.W., Hayes, K.C., de Witt, G.F., Jegathesan, M., Satgunasingham, N., Ong, A.S., and Tan, D.T.S. (1992) Palmitic Acid and Oleic Acid Exert Similar Effects on Plasma Lipid Profile in Normocholesterolemic Men and Women, *J. Am. Coll. Nutr. 11*, 383–390.
20. Sundram, K., Hayes, K.C., and Siru, O.H. (1994) Dietary Palmitic Acid Results in a Lower Serum Cholesterol Than a Lauric–Myristic Acid Combination in Normolipemic Humans, *Am. J. Clin. Nutr.* 59, 841–846.
21. Mattson, F.H., and Grundy, S.M. (1985) Comparison of the Effects of Dietary Saturated, Monounsaturated, and Polyunsaturated Fatty Acids and Lipoproteins in Man, *J. Lipid Res. 26*, 194–202.
22. Rudel, L.L., Haines, J.L., and Sawyer, J.K. (1990) Effect of Plasma Lipoproteins of Monounsaturated, Saturated, and Polyunsaturated Fatty Acid of African Green Monkeys, *J. Lipid Res. 31*, 1873–1882.
23. Khosla, P., and Hayes, K.C. (1993) Dietary Palmitic Acid Raises LDL Cholesterol Relative to Oleic Acid Only at a High Intake of Cholesterol, *Biochim. Biophys. Acta. 1210*, 13–22.
24. Hayes, K.C., and Khosla, P. (1992) Dietary Fatty Acid Thresholds and Cholesterolemia, *FASEB J. 6*, 2600–2607.
25. Fielding, C.J., Havel, R.J., Todd, K.M., Yeo, K.E., Schloetter, M.C., Weinberg, V., and Frost, P.H. (1995) Effects of Dietary Cholesterol and Fat Saturation on Plasma Lipoprotein in an Ethnically Diverse Population of Healthy Young Men, *J .Clin. Invest.* 95, 611–618.
26. Lepag, G., and Roy, C.C. (1986) Direct Transesterification of All Classes of Lipids in a One-Step Reaction, *J. Lipid Res.* 27, 114–120.
27. Markwell, M.A.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978) A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples, *Anal. Biochem. 87*, 206–210.
28. Chapman, M.J., Goldstein, S., Lagrange, D., and Laplaud, M.P. (1981) A Density Gradient Ultracentrifugal Procedure for the Isolation of the Major Lipoprotein Classes from Human Serum, *J. Lipid Res. 22*, 339–358.
29. Goulinet, S., and Chapman, M.J. (1993) Plasma Lipoproteins in Golden Syrian Hamster *(Mesocricetus auratus)*: Heterogeneity of apoB- and apoAI-Containing Particles, *J. Lipid Res. 34*, 943–959.
30. Hegsted, D.M., and Nicolosi, R.J. (1990). Do Formula Diets Attenuate the Serum Cholesterol Response to Dietary Fats? *J. Vas. Med. Biol. 2*, 68–73.
31. Goldberg, M.L., and Enig, M.G. (1993) Palmitic and Lauric Acids and Serum Cholesterol, *Am. J. Clin. Nutr. 58*, 244.
32. Hayes, K.C. (1993) Fatty Acids and Liquid-Formula Diets in Cholesterol Studies, *Am. J. Clin. Nutr. 57*, 231.
33. Zock, P.L., and Katan, M.B. (1992) Hydrogenation Alternatives: Effect of *trans* Fatty Acids and Stearic Acid Versus Linoleic Acid on Serum Lipid and Lipoproteins in Humans, *J. Lipid Res. 33*, 399–410.
34. Kritchevsky, D. (1988) Effect of Triglyceride Structure on Lipid Metabolism, *Nutr. Rev. 46*, 177–181.
35. Hayes, K.C. (1995) Saturated Fat and Blood Lipids: New Slant on an Old Story, *Can. J. Cardiol. 11*, 39G–46G.
36. Hayes, K.C., Pronczuk, A., and Khosla, P. (1995) A Rationale for Plasma Cholesterol Modulation by Dietary Fatty Acids: Modeling the Human Response in Animals, *J. Nutr. Biochem. 6*, 188–194.
37. Dietschy, J.M., Turley, S.D., and Spady, D.K. (1993) Role of the Liver in the Maintenance of Cholesterol and Low Density Lipoprotein Homeostasis in Different Animal Species, Including Humans, *J. Lipid Res. 34*, 1637–1659.
38. Khosla, P., and Hayes, K.C. (1991) Dietary Fat Saturation Rhesus Monkeys Affects LDL Concentrations by Modulating the Independent Production of LDL Apoprotein, *Biochim. Biophys. Acta 1083*, 46–56.
39. Khosla, P., and Hayes, K.C. (1992) Comparison Between Dietary Saturated (16:0) Monounsaturated (18:1) and Polyunsaturated (18:2) Fatty Acids on Plasma Lipoprotein Metabolism in Cebus and Rhesus Monkeys Fed Cholesterol-Free Diets, *Am. J. Clin. Nutr. 55*, 51–62.
40. Pronczuk, A., Patton, G.M., Stephan, Z.F., and Hayes, K.C. (1991) Species Variation in the Atherogenic Profiles of Monkeys: Relationship Between Dietary Fats, Lipids, and Platelet Aggregation, *Lipids 26*, 213–222.

[Received March 7, 1994, and in final revised form March 28, 1995; Revision accepted May 5, 1995]

Palmolein and Groundnut Oil Have Comparable Effects on Blood Lipids and Platelet Aggregation in Healthy Indian Subjects[1]

Ghafoorunissa*, Vinodini Reddy, and B. Sesikaran
National Institute of Nutrition, Hyderabad - 500 007, India

ABSTRACT: Substitution of palmolein (POL) for groundnut oil (GNO) doubles saturated fatty acids and decreases by half the linoleic acid (18:2n-6) content of Indian diets. The effects of this substitution on selected parameters of cardiovascular risk and membrane functions were studied in middle-aged subjects. Both metabolic (short-term) and "in-home" (long-term) studies were conducted, and the subjects were crossed over from GNO to POL or vice versa. During both studies and in both sexes, blood pressure, plasma levels of total cholesterol and triglycerides and their distributions in various lipoprotein fractions were not altered. The lower 18:2n-6 and higher 16:0 intakes were reflected in fatty acid compositions of cholesteryl esters and triglycerides. However, the plasma and platelet phospholipid fatty acid patterns did not shift toward saturation. The observation that the levels of long-chain polyunsaturated fatty acids in phospholipids were similar at the end of GNO and POL regimens indicates that 18:2n-6 furnished during POL regimen may be sufficient to maintain the levels of arachidonic acid in cell membranes. Platelet aggregation, erythrocyte membrane fluidity, and activity of Na^+,K^+ ATPase, a membrane-bound enzyme, were essentially similar at the end of the two oil regimens. These results indicate that POL is comparable to GNO and may not induce hypercholesterolemia in Indian subjects consuming cereal-based diets containing 30% total fat calories and low cholesterol.
Lipids 30, 1163–1169 (1995).

Diets high in fats rich in saturated fatty acids (SFA) raise plasma cholesterol, and are atherogenic and thrombogenic (1). Lauric (12:0), myristic (14:0), and palmitic (16:0) acids constitute the bulk of dietary SFA. These three do not contribute equally to hypercholesterolemia or hyperaggregability, and further, their effects vary with dietary levels of cholesterol and unsaturated fatty acids (2,3). Dietary fat also affects lipid compositions of membranes—alters their fluidity—and activities of receptors and membrane-bound enzymes (4–6). All these changes have pathophysiological significance. In most foods, 16:0 is the major SFA (7) and among oils, palm oil and palmolein (POL) have the highest levels (8). Therefore, there has been a debate on whether increased consumption of palm oil or POL could increase the risk of cardiovascular diseases (8–10). Consequently, several experimental (11–14) and human studies (15,16) were published on the effects of palm oil and POL on plasma lipids, and a more recent review has critically examined this subject (17). The present study was carried out to investigate the effects of substitution of GNO with POL in the habitual diets of Indian subjects on plasma lipids, platelet aggregation, erythrocyte membrane fluidity, and activity of erythrocyte Na^+,K^+ ATPase.

MATERIALS AND METHODS

Subjects and study design. Two studies were conducted in subjects drawn from the Institute staff. Criteria for subject selection included normal blood chemistry and no history of diabetes. Age, body weight, height, and blood pressure were recorded, and body mass index (kg/m^2) was calculated. A cross-over experimental design was used to control within subject variability and the effects of sequence of oil supplementation. Study 1 was short-term (metabolic study) in 12 men and Study 2 was a longer term ("in-home" study) of 12 men and 12 women.

Study 1. Individual diet surveys were done before starting the study using a 24-h recall oral questionnaire (18), and the total intake of various nutrients was calculated using food composition tables (19). During the study, the subjects were maintained on diets consisting of traditional menus prepared in the metabolic unit. The amount and type of foods as well as the daily menus were the same for all the subjects throughout the study, and only the cereal intake was altered to ensure satiety. In the first phase, half the subjects received refined groundnut oil (GNO) for 8 wk, and the other half received re-

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, September 1993.

*To whom correspondence should be addressed at National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania PO, Hyderabad - 500 007, India.

Abbreviations: EMR, estimated maximum response; ED_{50}, amount of aggregating agent required to bring about half the maximal response; GNO, groundnut oil; HDL, high density lipoproteins; LC, long chain; LDL, low density lipoproteins; MUFA, monounsaturated fatty acids; POL, palmolein; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; VLDL, very low density lipoproteins.

Copyright © 1995 by AOCS Press

fined, bleached, and deodorized POL imported from Malaysia. After a washout period of 6 wk, in the second 8-wk phase they were switched to the other oil regimen. Body weight was monitored at weekly intervals.

Study 2. This was a study of 12 men and 12 women conducted using the subjects' own home-prepared foods. The oil was supplied at monthly intervals with instructions to use it as the only cooking (added) fat source. During the first phase of the study, half the families were supplied GNO and the rest POL for 16 wk. During each phase of the study, 24-h recall diet surveys were done twice, and the subjects' mean daily intakes of macronutrients and fatty acids were calculated. In order to monitor compliance, the subjects were requested to record the total number of meals and snacks taken outside. The dietitian collected these records at 10-d intervals. Body weight was monitored every month.

Blood pressure. Blood pressure measurements were made after 10–12 h fasting using a calibrated conventional mercury sphygmomanometer, with the subject lying down. Recordings were made in duplicate and consistent values were taken.

Blood sampling. Fasting blood samples were collected at pre-entry and at the end of each oil regimen after ensuring that the subjects did not take anti-inflammatory drugs during the previous two weeks. For platelet aggregation studies, blood was collected in 3.8% trisodium citrate solution (9:1, vol/vol). For analysis of plasma lipids and for preparing erythrocyte membranes and platelet pellet, blood was collected in EDTA (1 mg/mL).

Platelet aggregation. Citrated blood was diluted with normal saline (1:1, vol/vol), and collagen-induced (1–10 μg/mL) whole blood aggregation was measured by the impedance method of Cardinal and Flower (20) using a Whole Blood Aggregometer, Model 530 (Chrono-log, Havertown, PA). Platelet-rich plasma (PRP) was obtained by spinning the citrated blood at $120 \times g$ for 10 min. Platelet-poor plasma (PPP) was obtained by spinning blood at $1000 \times g$ for 15 min. PRP was diluted with autologous PPP to obtain 2.5×10^5 cells/μL, and aggregation was measured in 0.25 mL PRP with varying concentrations (1–20 μM) of ADP by turbidometry (21). The primary aggregation rate was calculated for each concentration of aggregating agent, and the estimated maximum response (EMR) and dose of aggregating agent required to bring about half maximum response (ED_{50}) were calculated. For whole blood aggregation, lag phase also was calculated.

Lipids and fatty acids. The blood collected in EDTA was spun to obtain PRP. Platelet pellet and PPP were obtained by spinning PRP at $1000 \times g$ for 15 min. The pellet was washed with saline and the cells were lysed. Cholesterol and triglycerides in various lipoproteins and fatty acid composition of plasma were analyzed in PPP.

Plasma total cholesterol was estimated by the method of Watson (22). Total triglycerides were determined by the method of Lowell and Dunn (23). Lipoproteins were separated by the dual precipitation method of Wilson and Spiger (24). Cholesterol was assayed in each of the lipoprotein fractions, and triglycerides were estimated in very low density lipoprotein (VLDL) fraction.

Lipids were extracted from both PPP and lysed platelets with chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene and separated by thin-layer chromatography using hexane/diethylether/glacial acetic acid (73:25:2, by vol). The triglycerides, cholesteryl esters, and phospholipids were scraped into tubes and transmethylated as described earlier (25) along with 25 μg heptadecanoic acid (internal standard). The fatty acid methyl esters were analyzed by flame-ionization capillary gas–liquid chromatography using Perkin-Elmer 8500 Gas Chromatograph (Buckinghamshire, England) equipped with reintegration facility and GP 100 graphic printer. Supelco (Bellefonte, PA) fused silica SP™ 2330 capillary column 30 m × 0.25 mm id, 0.20 μm film thickness, was used. The column temperature was initially kept at 120°C for 8 min and then increased three times stepwise at 30°C/min to 150°C, at 10°C/min to 210°C and at 3°C/min to 235°C, then isothermally at 235°C for 10 min. The nitrogen pressure was maintained at 18 psi. Individual fatty acid methyl esters were identified by comparison of the retention times with authentic standards obtained from Nu-Chek-Prep (Elysian, MN) and Sigma Chemical (St. Louis, MO).

Erythrocyte membrane fluidity and Na^+,K^+ATPase activity. Erythrocyte membranes were prepared by the method of Hanahan and Ekholm (26). Membrane fluidity was monitored by measuring steady-state polarization of 1,6-diphenyl-1,3,5-hexatriene using a Hitachi 650-10S fluorescence spectrophotometer (Tokyo, Japan), and fluorescence anisotropy (rs) was calculated (27). Erythrocyte membrane protein was assayed by the method of Lowry *et al.* (28). Na^+,K^+ATPase activity was assayed according to Nakao *et al.* (29) using 200 μL (400–600 μg protein) erythrocyte membrane. Inorganic phosphate was estimated according to Fiske and Subbarow (30).

Statistical analysis. Results are given as mean ± standard error. The statistical significance between means (end of one oil period vs. end of second oil period) were analyzed using paired *t*-test; significant results were taken as $P < 0.05$. Two-way analysis of variance and "f" ratio test were used to evaluate the change in plasma total cholesterol between two oil periods vs. total cholesterol levels at pre-entry, and change in plasma triglycerides vs. body mass index at pre-entry. Unpaired *t*-test was done to compare the data between men and women.

RESULTS

The clinical characteristics of the subjects are given in Table 1. Men who volunteered for Study 2 were older and had higher plasma total cholesterol levels than subjects in Study 1. In Study 2, five subjects (three men and two women) had blood cholesterol values above 190 mg/dL, three of these subjects (two men and one woman) were also hypertensive; the medication for hypertension was not altered for these three subjects during the entire study.

TABLE 1
Clinical Description of the Subjects

Parameters before experiment	Study 1		Study 2			
	Men (n = 12)		Men (n = 12)		Women (n = 12)	
	Mean (SE)	Range	Mean (SE)	Range	Mean (SE)	Range
Age (years)	35 (1.1)	29–39	43 (1.6)	30–52	38 (1.3)	33–39
Body mass index[a]	21 (0.8)	18–27	23 (0.2)	17–26	24 (1.0)	16–30
Blood pressure (mm Hg)						
Systolic	112 (1.6)	100–120	111 (9.0)	110–150	116 (3.6)	102–140
Diastolic	79 (2.0)	70–84	83 (2.5)	70–102	83 (2.7)	72–102
Blood lipids (mg/dL)						
Total cholesterol	152 (5.2)	120–185	179 (9.5)	120–260	162 (6.6)	140–243
Total triglycerides	88 (11.1)	35–170	90 (15.3)	36–125	68 (5.7)	43–121

[a]Body mass index = weight (kg)/height^2 (m).

TABLE 2
Average Daily Intake of Major Food Groups and Macronutrients

Food groups[a] and macronutrients	Study 1 (men)		Study 2	
	Before experiment	During experiment	Men	Women
Cereals	260 ± 18	365 ± 16.5	255 ± 14	205 ± 9
Pulses	65 ± 7	75	55 ± 4	45 ± 4
Vegetables	220 ± 13	365	205 ± 8	170 ± 7
Milk and milk products	310 ± 35	315	410 ± 28	380 ± 33
Oil	32 ± 2.4	50	43 ± 3	38 ± 2
Ghee	4 ± 1.5	—	4 ± 2	2 ± 1
Nuts and oil seeds	15 ± 2.0	15	12 ± 2	12 ± 1
Flesh foods	75[b] ± 15	—	19[c] ± 7	16[c] ± 9
Macronutrients				
Total energy (Kcal)	2080 ± 75	2565 ± 25	2120 ± 91	1770 ± 75
Protein (en%)	13 ± 0.5	12	12 ± 0.9	13 ± 0.8
Total fat (en%)	27 ± 0.6	27	32 ± 0.8	32 ± 0.8
Fat from all food items (en%)	11.2 ± 0.6	9.5	11.5 ± 1.4	11.5 ± 0.7
Cooking oil (added) (en%)	15.6 ± 1.3	17.5	20.0 ± 2.6	20.3 ± 2.9
Cholesterol (mg)	85 ± 12	50	80 ± 6	71 ± 6

[a]Grams/person/day; values are mean ± SE; n = 12 in each group. [b]In five subjects only. [c]In six subjects only.

The average daily intakes of major food groups and macronutrients are presented in Table 2. In subjects of Study 1, the intake of various foods and total energy were higher during the study period as compared to the intakes on home (pre-entry) diets. In subjects of Study 2, the number of meals and snacks taken outside the home contributed less than 10% of the total number consumed during the 16-wk study period, and therefore the fat taken outside their homes was assumed not to have significantly contributed to the type of fat consumed. The total energy intake was lower in women than in men, but the percentage of total fat calories was similar.

The data on average daily intake of fatty acids during the two oil regimens is given in Table 3. In both the studies, use of POL provided twice as much 16:0 and half the quantity of linoleic acid (18:2n-6) than the GNO regimen. The ratios of polyunsaturated fatty acids (PUFA)/SFA and linoleic/α-linolenic acids (18:2n-6/18:3n-3) were low during the POL period.

In Study 1, five subjects gained weight during the study period (GNO = 3, POL = 2). In both studies, the mean body weights were similar at the end of the two oil regimens. Blood pressure, plasma total cholesterol, cholesterol in low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions, as well as total and VLDL triglycerides, were not altered by the type of oil consumed in both studies and for both

TABLE 3
Average Daily Intake of Fatty Acids from the Total Diet (en%)[a]

Fatty acids	Study 1		Study 2	
	GNO	POL	GNO	POL
Total SFA	8.2	12.2	11.4 ± 1.0	15.0 ± 0.7
16:0	4	9	6 ± 0.5	12 ± 0.8
18:0	1.2	1	2 ± 0.2	2 ± 0.2
Others[b]	3.0	2.2	3.4 ± 0.3	1.0 ± 0.2
Total MUFA	9.9	9.7	11.4 ± 1.2	11.7 ± 0.5
18:2n-6	6.9	3.2	7.1 ± 0.5	3.4 ± 0.2
18:3n-3	0.2	0.2	0.3 ± 0.02	0.2 ± 0.02
PUFA/SFA	0.8	0.3	0.6 ± 0.05	0.2 ± 0.01
18:2n-6/18:3n-3	33	15	30 ± 2.3	16 ± 1.3

[a]GNO, groundnut oil; MUFA, monounsaturated fatty acids; POL, palmolein; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; values are mean ± SE; n = 12 in each group.
[b]14:0, 20:0, 22:0, and 24:0.

TABLE 4
Body Weight, Blood Pressure, and Blood Lipids[a]

	Study 1		Study 2			
	Men		Men		Women	
Oils/parameters	GNO	POL	GNO	POL	GNO	POL
Body weight (kg)	61.3 ± 2	62 ± 2	62 ± 2	60 ± 2	60 ± 4	60 ± 3
Body mass index	21.6 ± 0.8	21.9 ± 0.8	22.9 ± 0.7	22.9 ± 0.7	24.6 ± 1.1	24.6 ± 1.0
Blood pressure						
Systolic	113 ± 3	109 ± 3	118 ± 8	119 ± 5	119 ± 4	117 ± 4
Diastolic	81 ± 3	77 ± 3	82 ± 2	82 ± 3	87 ± 3	86 ± 2
Blood lipids (mg/dL)						
Total cholesterol	150 ± 6.9	155 ± 8.7	182 ± 8.7	178 ± 5.7	168 ± 6.5	170 ± 3.9
LDL cholesterol	79 ± 4.5	71 ± 9.1	100 ± 7.1	104 ± 6.0	99 ± 6.7	98 ± 4.9
HDL cholesterol	34 ± 3.2	36 ± 3.8	33 ± 2.4	30 ± 2.3	29 ± 1.7	32 ± 1.5
Total triglycerides	90 ± 13.3	107 ± 21.8	81 ± 15.6	79 ± 12.0	74 ± 13.0	66 ± 5.5
VLDL triglycerides	55 ± 13	55 ± 15	42 ± 12.6	45 ± 9.5	36 ± 9.4	36 ± 3.9

[a]Values are mean ± SE; n = 12 in each group; paired *t*-test between oil periods—not significant; body mass index = weight (kg)/height^2 (m); HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoprotein. See Table 3 for other abbreviations.

sexes (Table 4). Whole blood aggregation in response to collagen (EMR, ED_{50}, and lag phase), as well as ADP-induced aggregation in PRP, were essentially similar at the end of the two oil regimens in both studies and for both sexes (Table 5). In Study 2, the basal values of EMR were higher in women than in men (Table 5), and erythrocyte membrane fluidity and Na^+,K^+ATPase activity were not altered by the type of oil consumed (Table 5).

The results of Study 1 on fatty acid composition of plasma lipids and platelet phospholipids are presented in Table 6. At the end of POL period, 18:2n-6 decreased in both triglycerides and cholesteryl esters and monounsaturated fatty acids (MUFA) increased in cholesteryl esters. Plasma and platelet phospholipid fatty acid compositions were essentially similar at the end of the two oil regimens. The results of Study 2 on fatty acid composition of plasma and platelet lipids (Table 7) show that in men, 18:2n-6 decreased in both triglycerides and cholesteryl esters, SFA increased in triglycerides, and MUFA increased in cholesteryl esters at the end of POL period. In women, these changes were small and did not achieve statistical significance. Plasma and platelet phospholipid fatty acid compositions were quite similar at the end of the two oil regimens for both sexes.

DISCUSSION

A substantial body of data indicates that dietary factors and genetic variability affect the risk of cardiovascular diseases (1). Among the dietary factors, SFA increase plasma cholesterol and platelet reactivity, and these changes in turn con-

TABLE 5
Functional Parameters of Cell Membranes[a]

	Study 1		Study 2			
	Men		Men		Women	
	GNO	POL	GNO	POL	GNO	POL
Platelet aggregation			Whole blood			
ED_{50} (μg collagen)	2.1 ± 0.2	2.1 ± 0.2	1.7 ± 0.1	2.1 ± 0.2	1.8 ± 0.1	1.9 ± 0.1
EMR (div/min)	34.0 ± 1.6	34.0 ± 1.7	31.0 ± 1.6[a]	30.0 ± 1.4[b]	43.0 ± 3.0[a]	44.0 ± 2.5[b]
Lag phase (s)						
2 μg collagen	120.0 ± 9.7	112.0 ± 6.2	103 ± 7.2	95 ± 10.7	95.0 ± 5.2	98.0 ± 9.9
5 μg collagen	66.0 ± 3.6	66.0 ± 3.3	67 ± 4.3	65 ± 4.0	59.0 ± 3.8	61.0 ± 5.3
			Platelet-rich plasma			
ED_{50} (μM ADP)	2.4 ± 0.3	2.6 ± 0.4	2.2 ± 0.2	2.3 ± 0.3	2.5 ± 0.3	1.9 ± 0.2
EMR (div/min)	67.0 ± 1.6	71.0 ± 4.0	71 ± 2.6	75 ± 3.5	77 ± 2.6	73 ± 2.8
			Erythrocyte membrane			
Na^+,K^+ ATPase (μmole Pi/h/mg)	—	—	0.13 ± 0.02	0.12 ± 0.01	0.14 ± 0.02	0.14 ± 0.02
Membrane fluidity (rs)	—	—	0.17 ± 0.004	0.17 ± 0.01	0.16 ± 0.007	0.17 ± 0.006

[a]Values are mean ± SE; n = 12 in each group. ED_{50}, dose of aggregating agent required to bring about half maximal response; EMR, estimated maximum response; similar superscripts given to mean values indicate significant differences $P < 0.01$ by Student's *t*-test between sexes. See Table 3 for other abbreviations.

TABLE 6
Fatty Acid Profile of Plasma and Platelet Lipids (n mole %) in an Eight-Week Study (Study 1)[a]

Plasma	GNO	POL
	Triglycerides (n = 6)	
Total SFA	36.8 ± 2.6	40.0 ± 2.5
16:0	30.0 ± 2.2	32.1 ± 2.9
18:0	4.7 ± 0.4	5.2 ± 0.5
Total MUFA	35.5 ± 1.2	37.0 ± 1.4
18:2n-6	22.0 ± 2.3[a]	15.0 ± 1.7[a]
18:3n-3	0.6 ± 0.09	1.0 ± 0.4
LC n-6 PUFA	3.9 ± 0.9	2.3 ± 0.5
LC n-3 PUFA	<0.5	<0.5
	Cholesteryl esters (n = 6)	
Total SFA	14.7 ± 0.7	17.2 ± 1.5
16:0	11.7 ± 0.5	12.3 ± 1.0
18:0	1.2 ± 0.2	1.4 ± 0.3
Total MUFA	20.2 ± 0.9[b]	24.8 ± 0.4[b]
18:2n-6	54.9 ± 1.6[a]	48.1 ± 2.7[a]
18:3n-3	<0.5	<0.5
LC n-6 PUFA	7.7 ± 0.6	8.0 ± 1.4
LC n-3 PUFA	<0.5	<0.5
	Phospholipids	
Total SFA	47.1 ± 2.0	46.3 ± 1.2
16:0	30.5 ± 1.8	31.3 ± 1.1
18:0	13.7 ± 0.4	12.6 ± 0.4
Total MUFA	12.4 ± 0.5	14.2 ± 0.6
18:2n-6	22.4 ± 1.2	20.9 ± 0.6
18:3n-3	<0.5	<0.5
LC n-6 PUFA	15.1 ± 1.2	15.7 ± 0.6
LC n-3 PUFA	2.9 ± 0.5	2.8 ± 0.4
	Platelet phospholipids	
Total SFA	44.0 ± 1.7	43.5 ± 1.0
16:0	20.9 ± 1.3	20.3 ± 1.1
18:0	19.3 ± 0.8	19.2 ± 0.8
Total MUFA	20.0 ± 0.6	19.9 ± 0.5
18:2n-6	7.1 ± 0.6	6.6 ± 0.5
18:3n-6	0.8 ± 0.07	0.7 ± 0.08
LC n-6 PUFA	24.0 ± 0.9	26.1 ± 0.5
LC n-3 PUFA	3.5 ± 0.5	3.8 ± 0.7

[a]Values are mean ± SE; n = 12 in each group except where indicated in parentheses; LC, long chain; see Table 3 for other abbreviations; LC n-3 PUFA include 20:5, 22:5, and 22:6; SFA other than 16:0 and 18:0 include 20:0, 22:0, and 24:0; total MUFA—mainly 18:1; similar superscripts given to mean values indicate significant differences a = $P < 0.02$; b = $P < 0.01$ by paired *t*-test.

tribute to atherosclerosis and thrombosis (1). This study describes the effects of substituting GNO with POL in diets of middle-age Indian subjects on various parameters which increase the risk of cardiovascular diseases. Body weight can affect both blood pressure and serum lipids. Since the energy intakes and body weights were similar at the end of the two oil regimens, the results presented here can be attributed to differences in the type of oil consumed. In Study 1, body weights at pre-entry and at the end of each oil regimen were similar despite lower (~16%) total energy intakes at pre-entry. It is possible that the 24-h recall method has given underestimated values of energy intakes.

Substitution of POL for GNO doubles the 16:0 and reduces by half the 18:2n-6 content of cereal based diets containing about 30% total fat calories. Despite this, blood pressure, serum total cholesterol, and cholesterol distribution in various lipoproteins and total and VLDL triglycerides were not altered over 16 wk. It has been reported that 18:2n-6 reduces blood pressure in hypertensive but not in normotensive subjects (31). The lack of 18:2n-6 effect on blood pressure observed in this study may be because all the subjects (except three) were normotensive. Further, the observations made here suggest that increasing 16:0 up to about 12 en% does not raise blood pressure.

The effects of POL observed in this study are consistent with other studies showing that palm oil does not raise serum

TABLE 7
Fatty Acid Profile of Plasma and Platelet Lipids (n mole %) in a 16-Week Study (Study 2)[a]

	Men		Women	
Plasma	GNO	POL	GNO	P.OL
	Triglycerides (n = 9)			
Total SFA	35.0 ± 2.2[b]	42.8 ± 3.9[b]	34.7 ± 2.0	35.2 ± 1.4
16:0	31.1 ± 1.9	37.5 ± 3.3	29.8 ± 2.1	31.3 ± 1.6
18:0	3.9 ± 0.6	5.2 ± 1.2	4.8 ± 0.8	3.3 ± 0.4
Total MUFA	36.7 ± 1.2	33.1 ± 3.2	35.3 ± 2.6	39.6 ± 2.0
18:2n-6	24.8 ± 2.0[a]	20.2 ± 2.4[a]	23.8 ± 2.6	22.7 ± 2.1
18:3n-3	<0.5	<0.5	<0.5	<0.5
LC n-6 PUFA	2.2 ± 0.6	2.8 ± 0.7	3.3 ± 0.8	1.8 ± 0.2
LC n-3 PUFA	<0.5	<0.5	<0.5	<0.5
	Cholesteryl esters (n = 9)			
Total SFA	16.5 ± 1.7	21.0 ± 2.1	20.9 ± 2.0	20.2 ± 1.8
16:0	13.3 ± 1.0	17.6 ± 2.0	17.8 ± 2.0	16.8 ± 1.3
18:0	3.2 ± 0.7	4.4 ± 0.9	2.7 ± 0.5	3.3 ± 0.7
Total MUFA	23.0 ± 1.5[a]	28.6 ± 1.2[a]	25.5 ± 1.8	27.6 ± 1.7
18:2n-6	54.8 ± 2.5[c]	44.7 ± 3.4[c]	46.4 ± 3.8	44.4 ± 3.4
18:3n-3	<0.5	<0.5	<0.5	<0.5
LC n-6 PUFA	5.6 ± 0.8	5.2 ± 0.4	6.4 ± 0.5	6.3 ± 0.7
LC n-3 PUFA	<0.5	<0.5	<0.5	<0.5
	Phospholipids			
Total SFA	44.6 ± 1.5	45.0 ± 0.8	45.1 ± 0.7	46.2 ± 1.1
16:0	27.9 ± 1.1	29.2 ± 0.8	27.4 ± 0.8	30.3 ± 1.3
18:0	13.3 ± 0.6	12.9 ± 0.4	13.7 ± 0.4	13.4 ± 0.4
Total MUFA	14.7 ± 0.8	14.4 ± 0.6	14.1 ± 0.5	13.3 ± 0.7
18:2n-6	23.2 ± 1.1	21.4 ± 1.0	20.8 ± 1.1	20.3 ± 0.6
18:3n-3	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
LC n-6 PUFA	12.2 ± 0.6	13.3 ± 1.0	12.2 ± 0.6	13.2 ± 0.7
LC n-3 PUFA	1.7 ± 0.3	1.6 ± 0.1	2.7 ± 0.4	2.3 ± 0.2
	Platelet phospholipids			
Total SFA	44.6 ± 0.9	43.2 ± 0.9	44.2 ± 0.7	44.9 ± 1.1
16:0	18.3 ± 0.9	18.7 ± 0.7	18.3 ± 0.6	19.2 ± 0.8
18:0	18.1 ± 0.4	18.0 ± 0.5	18.8 ± 0.5	18.8 ± 0.4
Total MUFA	18.9 ± 0.3	18.1 ± 1.3	19.2 ± 0.7	19.4 ± 1.0
18:2n-6	8.6 ± 0.5	8.5 ± 0.4	8.3 ± 0.5	7.3 ± 0.4
18:3n-3	1.6 ± 0.2	2.0 ± 0.3	1.7 ± 0.3	1.4 ± 0.2
LC n-6 PUFA	22.9 ± 1.2	22.0 ± 1.1	23.1 ± 0.9	22.5 ± 0.9
LC n-3 PUFA	2.8 ± 0.4	2.7 ± 0.3	2.7 ± 0.4	2.6 ± 0.4

[a]Values are mean ± SE; n = 12 in each group except where indicated in parentheses; see Table 3 and 6 for abbreviations; LC n-3 PUFA includes 20:5, 22:5, and 22:6; LC n-6 PUFA includes 20:3, 20:4, and 22:5; SFA other than 16:0 and 18:0 include 20:0, 22:0, and 24:0; total MUFA—mainly 18:1; similar superscripts given to mean values indicate significant differences a = $P < 0.01$; b = $P < 0.02$; c = $P < 0.05$ by paired *t*-test.

cholesterol (11–17). Some recent studies have reported that 16:0 has no effect on serum cholesterol in normocholesterolemic subjects consuming diets containing <300 mg cholesterol, whereas it raises serum cholesterol in hypercholesterolemic subjects consuming >400 mg cholesterol per day (3,32). However, the cholesterol intake in the subjects studied here was low (80 mg/d) and in 80% of the subjects the serum cholesterol levels at pre-entry were below 220 mg/dL. Further, the change in serum cholesterol at the end of POL period was not related to the cholesterol level at pre-entry. In normocholesterolemic subjects consuming diets low in 14:0 and cholesterol, the effects of 16:0 were found to be comparable to 18:1 on serum total cholesterol and LDL/HDL ratio (33). In the present study, the dietary 14:0 was low and 18:1 levels were similar during the two oil regimens. However, 18:2n-6 levels increased from 3.3 to 7 en% during POL and GNO regimens, respectively. It has been suggested that in healthy subjects, as long as the percent energy from 18:2n-6 exceeds 2.5, 16:0 does not raise serum cholesterol, whereas stressed populations have a threshold of 18:2n-6 near 5–6 en% (32). The lack of 18:2n-6 effect (i.e., no change in serum cholesterol despite increasing dietary 18:2n-6 by 3.7 en% observed here) appears to be the result of low 18:2n-6 threshold in these subjects.

The fatty acid composition of plasma and membrane lipids reflects the overall balance of fatty acid intakes and metabolic regulations. In men (but not in women), the lower 18:2n-6 and higher 16:0 intakes during POL regimen were reflected in the compositions of triglycerides and cholesteryl esters. The differences in the response to dietary fatty acids observed between sexes cannot be due to compliance since the subjects of both sexes (Study 2) were drawn from the same family and consumed the same meals. A higher proportion of 18:2n-6 in plasma triglycerides and erythrocyte membranes in Asian diabetics as compared to Europeans has been attributed to differences in dietary levels of 18:2n-6, but sex difference was not observed in the tissue response to dietary fatty acids (34).

Studies in which dairy fat consumption was increased indicate that the markers of high SFA intake are MUFA and eicosatrienoic acid (20:3n-9) in plasma and platelet phospholipids (35). The data presented here (Tables 6 and 7) show that the plasma and platelet phospholipid fatty acid compositions were essentially similar at the end of the two oil periods. However, the high SFA intake during POL regimen was reflected in the levels of MUFA in cholesteryl esters. It appears that about 3 en% 18:2n-6 may be sufficient to maintain membrane levels of arachidonic acid. This fatty acid being the predominant PUFA plays an important role in both eicosanoid and noneicosanoid functions of membranes (4,7).

The risk factors of cardiovascular disease, including high dietary SFA, may increase platelet aggregation which, in turn, participates in atherosclerosis and thrombosis (1). A positive relation has been shown between diet rich in SFA and *ex vivo* platelet aggregability to most agonists (35,36). It has been suggested that ED_{50} and EMR give a better description of platelet function than measurements made with a particular dose of agonist (21,37), and aggregation in whole blood using impedance is more sensitive to antiplatelet drugs than the measurement made in PRP (38). In the present study, the EMR and ED_{50} measurements were done in both PRP and whole blood, although in response to different aggregating agents. The data presented in Table 5 shows that substitution of GNO with POL does not affect the aggregability of platelets. This could be because the platelet arachidonic acid levels, which are known to be strong determinants of *in vitro* platelet reactivity were similar during the two oil regimens. Qureshi *et al.* (39) reported that dietary tocotrienols from palm oil (Palmvitee) reduce *ex vivo* thromboxane B_2 generation and platelet aggregation. It was subsequently reported that (40) tocotrienol-rich vitamin E from palm oil did not alter thromboxane A_2, prostacyclin I_2, and platelet aggregation. However, in the present study, we have not measured thromboxane A_2 and prostacyclin. The high EMR for collagen-induced whole blood aggregation (Table 5) in women indicates greater aggregability as compared to men, but ED_{50} and lag phase in whole blood and ED_{50} and EMR in PRP were similar for both genders. The reasons for this discrepancy are not clear.

The lipid composition and functions of erythrocyte membranes can be modified by the type of dietary fat (4,5). In the present study, although the fatty acid composition of erythrocyte membranes was not measured, fluidity and activity of Na^+,K^+ATPase, a membrane-bound enzyme, were determined. That POL did not affect these parameters suggests either that the fatty acid composition of erythrocytes was not altered or the magnitude of changes were not sufficient to alter these membrane functions.

This study assessed the effects of substitution of GNO with POL in cereal-based diets which contained low cholesterol and about 30% total fat calories on blood lipids, platelet aggregation, and erythrocyte membrane functions of normal Indian subjects. The failure to demonstrate changes in the above parameters in 12 men and 12 women during 8- or 16-wk cross-over periods for each fat suggests that POL is comparable to GNO and does not induce the adverse effects usually associated with dietary saturated fat. However, to draw definitive conclusions it will be necessary to extend these studies to a larger number of subjects with one or more risk factors (hyperinsulinemia, obesity, hypertension) for cardiovascular disease.

ACKNOWLEDGMENTS

The financial support from Palm oil Research Institute, Malaysia (PORIM), is gratefully acknowledged. The authors thank Vani Acharya, E.P. Ramchandran, and Laxmi Rajkumar for technical help and Verghese for secretarial assistance.

REFERENCES

1. Ashwell, M. (1993) *Diet and Heart Disease. A Round Table of Factors,* British Nutrition Foundation, London.
2. Hayes, K.C., Pronezuk, A., Lindsey, S., and Diersen-Schade, D. (1991) Dietary Saturated Fatty Acids (12:0, 14:0, 16:0) Differ

in Their Impact on Plasma Cholesterol Lipoproteins in Nonhuman Primates, *Am. J. Clin. Nutr. 53*, 491–498.
3. Khosla, P., and Hayes, K.C. (1994) Cholesterolaemic Effects of the Saturated Fatty Acids of Palm Oil, *Food and Nutr. Bull. 15*, 119–125.
4. Edwards Webb, J.D., and Gurr, M.I. (1988) The Influence of Dietary Fats on the Chemical Composition and Physical Properties of Biological Membranes, *Nutr. Res. 8*, 1297–1305.
5. Vajreswari, A., and Narayana Reddy, K. (1992) Effect of Dietary Fats on Erythrocyte Membrane Lipid Composition and Membrane Bound Enzyme Activities, *Metabolism 41*, 352–358.
6. Spady, D.K., Woollet, L.A, and Dietschy, J.M. (1993) Regulation of Plasma LDL-Cholesterol Levels by Dietary Cholesterol and Fatty Acids, *Annu. Rev. Nutr. 13*, 355–381.
7. The Report of the British Nutrition Foundation's Task Force (1992) *Unsaturated Fatty Acids. British Nutrition Foundation. Nutrition and Physiological Significance*, British Nutrition Foundation, London.
8. Cottrell, R.C. (1991) Introduction: Nutritional Aspects of Palm Oil, *Am. J. Clin. Nutr. 53*, 989S–1009S.
9. James, J.M. (1989) Tropical Oils: Truth and Consequences, *Cereal Foods World 34*, 866–871.
10. Sugano, M. (1987) One Counter Argument to the Theory That Tropical Oils Are Harmful, *Yakagaku (J. Jap. Oil Chem. Soc.) 40*, 48–51.
11. Khor, H.T., and Tan, D.T.S. (1992) Studies on the Lipidemic Property of Dietary Palm Oil: Comparison on the Responses of Serum, Liver and Heart Lipids to Dietary Palm Oil, Palm Oil Triglycerides, Coconut Oil and Olive Oil, *Nutr. Res. 12 (Suppl. 1)*, S105–S115.
12. Sugano, M., and Imaizumi, K. (1991) Effect of Palm Oil on Lipid and Lipoprotein Metabolism and Eicosanoid Production in Rats, *Am. J. Clin. Nutr. 53*, 1034S–1038S.
13. Manorama, R., and Rukmini, C. (1992) Serum Lipids of Rats Fed Crude and Refined Palm Oil in High Cholesterol and Cholesterol Free Diets, *Nutr. Res. 12 (Suppl. 1)*, S93–S103.
14. Sundram, K. Khor, H.T., and Ong, A.S.H. (1990) Effect of Dietary Palm Oil and Its Fractions on Rat Plasma and High Density Lipoprotein Lipids, *Lipids 25*, 187–193.
15. Heber, D., Ashley, J.M., Solares, M.E., Wang, H.J., and Alfin-Slater, R.B. (1992) The Effects of Palm-Oil Enriched Diet on Plasma Lipids and Lipoproteins in Healthy Young Men, *Nutr. Res. 12 (Suppl. 1)*, S53–S59.
16. Ng, T.K.W., Hassan, K., Lim, J.B., Lye, M.S., and Ishak, R. (1991) Non Hyper-Cholesterolaemic Effects of a Palm Oil Diet in Malaysian Volunteers, *Am. J. Clin. Nutr. 53*, 1015S–1020S.
17. Ng, T.K.W. (1994) A Critical Review of the Cholesterolaemic Effects of Palm Oil, *Food and Nutr. Bull. 15*, 112–118.
18. Thimmayamma, B.V.S. (1987) *A Handbook of Schedules and Guidelines in Socioeconomic and Diet Surveys*, National Institute of Nutrition, Hyderabad.
19. Gopalan, C., Ramasastry, B.V., and Balasubramanian, S.C., revised and updated by Narasinga Rao, B.S., Deosthale, Y.G., and Pant, K.C. (1989) *Nutrient Composition of Indian Foods*, 2nd edn., ICMR offset press, New Delhi.
20. Cardinal, D.L., and Flower, R.J. (1980) The Electronic Aggregometer. A Noval Device for Assessing Platelet Behaviour in Blood, *J. Pharmacol. Meth. 3*, 135–158.
21. Thompson, S.G., and Vickers, M.V. (1985) Method in Dose Response Platelet Aggregometry, *Thromb. Haemostasis. 5*, 216–218.
22. Watson, D. (1960) A Simple Method for the Determination of Serum Cholesterol, *Clin. Chim. Acta. 5*, 637–643.
23. Lowell, B.F., and Dunn, R.F. (1973) Stable Reagent for Determination of Serum Triglycerides by Colorimetric Hantzsch Condensation Method, *Clin. Chem. 19*, 338–340.
24. Wilson, D.E., and Spiger, M.J. (1973) A Dual Precipitation Method for Quantitative Plasma Lipoprotein Measurement Without Ultracentrifugation, *J. Lab. Clin. Meth. 82*, 473–482.
25. Ghafoorunissa (1987) Effect of Edible Oils on Essential Fatty acid Nutriture of Rats, *J. Clin. Biochem. Nutr. 3*, 47–54.
26. Hanahan, D.J., and Ekholm, J.E. (1974) *Methods in Enzymology, 31, Part A*, 168–172.
27. Shinitzky, M., and Barenholz, Y. (1978) Fluidity Parameters of Lipid Regions Determined by Fluorescence Polarization, *Biochim. Biophys. Acta. 515*, 367–394.
28. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
29. Nakao, K., Kurashina, S., and Nakao, M. (1967) Adenosinetriphosphatase Activity of Erythrocyte Membrane in Hereditary Spherocytosis, *Life Sciences 6*, 595–600.
30. Fiske, C.H., and Subbarow, Y. (1925) The Colorimetric Determination of Phosphorus, *J. Biol. Chem. 66*, 375–380.
31. Iacono, J.M., and Dougherty, R.M. (1993) Effects of Polyunsaturated Fats on Blood Pressure, *Annu. Rev. Nutr. 13*, 243–260.
32. Hayes, K.C., and Khosla, P. (1992) Dietary Fatty Acid Thresholds and Cholesterolaemia, *FASEB J. 6*, 2600–2607.
33. Sundram, K., Hayes, K.C., and Siru, O.H. (1995) Both Dietary 18:2 and 16:0 May Be Required to Improve the Serum LDL/HDL Cholesterol Ratio in Normocholesterolemic Men, *Nutr. Biochem. 6*, 179–187.
34. Peterson, D.B., Fisher, K., Carter, R.D., and Mann, J. (1994) Fatty Acid Composition of Erythrocytes and Plasma Triglyceride and Cardiovascular Risk in Asian Diabetic Patients, *The Lancet 343*, 1528–1530.
35. Renaud, S., Backer, D.E.G., Thevenon, C., Joossens, J.V., Vermylen, J., Kornitzer, M., and Verstraetes, M. (1991) Platelet Fatty Acids and Function in Two District Regions of Belgium:relationship to Age and Dietary Habits, *J. Intern. Med. 229*, 79–88.
36. Renaud, S., Godsey, F., Dumont, E., Thevenon, C., Ortchanian, E., and Martin, J.L. (1986) Influence of Long-Term Diet Modification on Platelet Function and Composition in Moselli Farmers, *Am. J. Clin. Nutr. 43*, 136–150.
37. Meade, T.W., Vickers, M.V., Thompson, S.G., Stirling, Y., Haines, A.P., and Miller, G.J. (1985) Epidemiological Characteristics of Platelet Aggregability, *Br. Med. J. 290*, 428–432.
38. Riess, H., Braun, G., Brehm, G., and Hiller, E. (1986) Critical Evaluation of Platelet Aggregation in Whole Human Blood, *Am. J. Clin. Pathol. 85*, 50–56.
39. Qureshi, A.A., Qureshi, N., Wright, J.J.K., Shen, Z., Kramer, G., Gapor, A., Chong, Y.H., Dewitt, G., Ong, A.S.H., Peterson, D.M., and Bradlow, B.A. (1991) Lowering of Serum Cholesterol in Hypercholesterolemic Humans by Tocotrienols (Palm Vitee), *Am. J. Clin. Nutr. 53*, 1021S–1026S.
40. Wahlqvist, M., Krivokuca-Bogetic, Z., Lo, C.S., Hage, B., Smith, R., and Lukito, W. (1992) Differential Serum Responses of Tocophenols and Tocotrienols During Vitamin Supplementation in Hypercholesterolemic Individuals Without Changes in Coronary Risk Factors, *Nutr. Res. 12 (Suppl. 1)*, S181–S201.

[Received December 23, 1993, and in final revised form October 9, 1995; Revision accepted October 10, 1995]

Response of Hypercholesterolemic Subjects to Administration of Tocotrienols[1]

A.A. Qureshi[a,*], B.A. Bradlow[b], L. Brace[b], J. Manganello[b], D.M. Peterson[c], B.C. Pearce[d], J.J.K. Wright[d], A. Gapor[e], and C.E. Elson[f]

[a]Advanced Medical Research, Madison, Wisconsin 53719, [b]Department of Pathology, University of Illinois College of Medicine, Chicago, Illinois 60612, [c]Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492, [d]Cereal Crops Research Unit, USDA, Madison, Wisconsin 53705, [e]Palm Oil Research Institute of Malaysia, Kuala Lumpur, Malaysia and [f]Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT: The cholesterol-suppressive actions of Palmvitee and γ-tocotrienol were assessed in hypercholesterolemic subjects after acclimation to the American Heart Association Step 1 dietary regimen for four and eight weeks, respectively. The four-week dietary regimen alone elicited a 5% decrease ($P < 0.05$) in the cholesterol level of the 36 subjects. Subjects continuing on the dietary regimen for a second four-week period experienced an additional 2% decrease in their cholesterol levels. Dietary assessments based on unanticipated recalls of 24-h food intake records suggest that significant reductions in energy and fat, predominantly in saturated fat, intakes are responsible. The subjects experienced significant Palmvitee- and γ-tocotrienol-mediated decreases in cholesterol. The group of subjects acclimated to the dietary regimen for four weeks responded to Palmvitee (a blend of tocols providing 40 mg α-tocopherol, 48 mg α-tocotrienol, 112 mg γ-tocotrienol, and 60 mg δ-tocotrienol/day for four weeks) with a 10% decrease in cholesterol ($P < 0.05$). Dietary assessments showed no further change in energy and fat intakes. α-Tocopherol attenuates the cholesterol-suppressive action of the tocotrienols. The second group of subjects, acclimated to the dietary regimen for eight weeks, received 200 mg γ-tocotrienol/d for four weeks. The cholesterol-suppressive potency of this α-tocopherol-free preparation was calculated to be equivalent to that of the mixture of tocotrienols (220 mg) used in the prior study. Cholesterol levels of the 16 subjects in the second group decreased 13% ($P < 0.05$) during the four-week trial. Plasma apolipoprotein B and *ex vivo* generation of thromboxane B_2 were similarly responsive to the tocotrienol preparations, whereas neither preparation had an impact on high density lipoprotein cholesterol and apolipoprotein A-I levels.

Lipids 30, 1171–1177 (1995).

The tocotrienols initiate post-transcriptional actions which lead to the down regulation of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase activity (1). Mevalonic acid, the product of this reaction, is generally considered to be the rate-limiting substrate for the synthesis of cholesterol. A decrease in serum cholesterol, a response consistent with the tocotrienol-mediated down-regulation of HMG–CoA reductase activity, is reported from some (2,3), but not all, human studies (4). We (2) and others (3,4) have reported the failure of some individual hypercholesterolemic subjects to respond to the tocotrienols. This variation could be anticipated, as the biological action of tocotrienols may limit their impact to those subjects who have an overproduction of cholesterol.

Excessive dietary intake of cholesterol overrides the biological action of the tocotrienols, but other confounding factors may be less apparent. Subjects receiving placebos often record modest decreases in blood lipids; these changes are attributed to subtle changes in dietary patterns. We recently confirmed (5) our preliminary finding (6) that α-tocopherol attenuates the impact of the tocotrienols on HMG–CoA reductase activity. This finding addresses another confounding factor. Tocotrienol preparations available for human tests (Palmvitee; BCP, AG; PORIM, Kuala Lumpur, Malaysia) are not standardized. Our review of the tocotrienol literature found that the studies showing a cholesterol-lowering action of the tocotrienols employed either a purified tocotrienol or Palmvitee preparations containing less than 20% tocopherol (5). The following study was designed to avoid the aforementioned confounding factors.

METHODS

Study population. Subjects were recruited from a hypercholesterolemic population (serum cholesterol > 5.7 mmol/L) screened at the University of Illinois, Chicago (Chicago, IL). Volunteers were excluded on the basis of weight (>150% of Metropolitan Life ideal weight), use of cholesterol-altering medication, an elevated serum glutamate-pyruvate or glutamate-oxaloacetate transaminase activity, an elevated blood urea nitrogen or glucose level, diabetes, or a history of a liver, renal, or hypertensive disease. Subject screening was accomplished during a three-week period. A fasting blood sample

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, September 1993.

*To whom correspondence should be addressed at Advanced Medical Research, 8251 Raymond Road, Madison, WI 53719.

Abbreviations: AHA Diet, American Heart Association Step 1 Diet; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; HDL, high density lipoprotein; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; LDL, low density lipoprotein; TxB_2, thromboxane B_2

Copyright © 1995 by AOCS Press

was collected for cholesterol determination at the initial session following the determination of eligibility. Following blocking by gender and stratification according to low and high cholesterol levels as determined at screening, the subjects in each of the four subsets were randomized into an experimental (Palmvitee) group of 20 subjects (7.02 ± 0.99 mmol cholesterol/L) and a control (Placebo) group of 16 subjects (7.32 ± 0.84 mmol cholesterol/L). The groups were uniform in male/female distribution (45:55), age (40.6 ± 12.1 y), and body mass index (28.1 ± 4.9). All subjects signed an informed consent which was approved by the University of Illinois, Chicago Medical Center Institutional Review Board. The study was conducted under IND number 30906.

Experimental design. The 16-wk study consisted sequentially of Baseline, American Heart Association Diet (7,8), Palmvitee, and γ-Tocotrienol phases.

Baseline phase. On entry, the subjects met individually and in small group sessions with counselors. The individual sessions focused on 24-h recalls of food consumption, and the group sessions provided instructions for keeping three-day records of food intake (two weekdays, one weekend day). Subjects were encouraged to follow their typical dietary pattern and were instructed to keep food records for the terminal three days of this and the following phases. Subjects also received an unanticipated telephone call for a 24-h recall of food intake.

American Heart Association Step 1 Diet (AHA Diet) phase. Subjects met in small groups for discussions of the relationship between diet and cardiovascular risk factors and for instruction on the AHA Diet. Each subject received a copy of the *1988 AHA Diet, Patient Manual*, and the telephone number of a staff contact person.

Palmvitee phase. The subjects were continued on the AHA Diet during this phase; each subject consumed four 300 mg capsules daily. Subjects in the Placebo group received capsules containing 300 mg corn oil and ~0.25 mg α-tocopherol. Palmvitee subjects received capsules containing 235 mg palm olein, 10 mg α-tocopherol, 12 mg α-tocotrienol, 28 mg γ-tocotrienol, and 15 mg δ-tocotrienol (AG; PORIM, Kuala Lumpur, Malaysia).

γ-Tocotrienol phase. The 16 Placebo subjects were continued on the AHA Diet regimen for a final four weeks, during which each received four 300 mg capsules daily (each capsule contained 250 mg palmolein and 50 mg γ-tocotrienol). γ-Tocotrienol was isolated the tocotrienol-rich fraction of palm oil (AG; PORIM).

Analyses. Initial measures include the subjects' height, weight, blood pressure, history of significant diseases, medications, and alcohol use. Weights were recorded weekly. Venous blood samples were drawn at 7:00–9:00 a.m. following an overnight fast at the Baseline phase (week three represents data obtained at screening and at week four), and at weeks seven and eight (AHA Diet), 11 and 12 (Palmvitee), and 15 and 16 (γ-Tocotrienol) of the study. Processed samples were held at −72°C until analysis.

The study design provided control and treatment cholesterol values for each subject. Automated clinical laboratory procedures were used for cholesterol determinations at weeks 4, 8, 12, and 16. We found that the standard deviations for the paired differences were on the order of 51 ± 12% of the mean. At the termination of the study, we analyzed the samples according to the manual protocol. The standard deviations for the paired differences were on the order of 15 ± 3% of the mean (from Scheme 1). We then sent random coded samples to clinical laboratories at the University of Illinois and the University of Wisconsin for analyses. The results of these analyses proved to be more consistent with those obtained by the manual protocol. The manual and automated clinical procedures yielded similar values for the subjects with the relatively lower cholesterol levels. It should be noted that the latter clinical determinations were run with instruments at each setting calibrated against a single standard, whereas the ini-

	Baseline	Δ1	AHA Diet	Δ2	AHA Diet	Δ3	γ-Tocotrienol
Group							
Placebo	7.29 ± 0.72		6.89 ± 0.82		6.69 ± 0.83		5.82 ± 0.69
Paired difference		−0.40 ± 0.07		−0.20 ± 0.03		−0.87 ± 0.10	

Δ1 > Δ2 $P < 0.025$ Δ2 < Δ3 $P < 0.001$

	Baseline	Δ1	AHA Diet	Δ2	Palmvitee
Palmvitee	7.03 ± 0.06		6.65 ± 1.04		5.97 ± 1.01
Paired difference		−0.38 ± 0.05		−0.68 ± 0.12	

Δ1 < Δ2 $P < 0.050$

SCHEME 1

TABLE 1
Impact of Four-Week American Heart Association Step 1 Diet (AHA) Regimen on Dietary Intake[a]

Dietary regimen	n	Baseline	AHA	*t*	*P*
Energy (kJ/d)	36	7498 ± 3367	5807 ± 1898	2.96	0.01
Protein (g/d)	36	71.7 ± 33.4	65.6 ± 28.5	0.83	NS
Carbohydrate (g/d)	36	221.9 ± 87.8	178.9 ± 74.1	2.23	0.05
Fat (g/d)	36	67.6 ± 46.0	47.3 ± 25.0	2.32	0.03
SFA (g/d)	36	23.6 ± 7.9	14.6 ± 8.5	4.66	0.01
MUFA (g/d)		24.9 ± 17.6	16.9 ± 16.4	1.96	0.07
PUFA (g/d)		12.6 ± 9.3	11.4 ± 5.9	0.65	NS
Cholesterol (mg/d)	36	220 ± 170	148 ± 80	2.29	0.04
Alcohol (g/d)	36	4.4 ± 11.1	0.6 ± 2.2	2.01	0.06
Fiber (g/d)	36	14.0 ± 6.6	15.2 ± 6.9	0.75	NS

[a]SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; NS, not significant; *t*, paired two-failed *t*-test, *P*, probability.

TABLE 2
Impact of Four-Week American Heart Association Step 1 Diet (AHA) Regimen on Blood Parameters[a]

Parameter	n	Baseline	AHA	*t*	*P*
Cholesterol (mmol/L)	36	7.15 ± 0.85	6.76 ± 0.94	2.50	0.03
LDL cholesterol (mmol/L)	36	5.10 ± 0.80	4.69 ± 0.86	2.07	0.05
ApoB (g/L)	36	1.00 ± 0.12	0.91 ± 0.09	3.60	0.01
HDL cholesterol (mmol/L)	36	1.13 ± 0.20	1.15 ± 0.18	0.45	NS
ApoA-I (g/L)	36	1.12 ± 0.10	1.13 ± 0.11	0.40	NS
Triglycerides (mmol/L)	36	1.93 ± 0.44	1.86 ± 0.43	0.68	NS
Glucose (mmol/L)	36	5.97 ± 0.86	5.66 ± 0.77	1.63	NS

[a]LDL, low density lipoprotein; ApoB, apolipoprotein B; HDL, high density lipoprotein; ApoA-I, apolipoprotein A-I; NS, not significant. See Table 1 for other abbreviations.

tial analyses were conducted over a nine-month period with multiple standards. Alternatively, the extended incubation employed with the manual procedure might permit the more accurate determination of very elevated cholesterol levels.

The analyses of the coded samples were performed at Advanced Medical Research (Madison, WI). Serum cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride, and glucose concentrations were estimated with reagent kits from Sigma Chemical Co. (St. Louis, MO). The incubation time for the cholesterol determinations was 10 min. Serum apoprotein A-I (ApoA-I), apolipoprotein B (ApoB), and thromboxane B_2 (TxB_2) concentrations were determined by radio immunoassay using kits from Sigma (ApoA-I, ApoB) and Chemicon International Chemicon International (El Segundo, CA) (TxB_2).

Diet records and 24-h recalls were analyzed (Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN); if required, subjects were individually counseled to modify food intake to meet the goals of the AHA Diet or to maintain weight.

Statistical analyses. Treatment effects were evaluated by analysis of variance (SuperANOVA; Abacus Concepts, Berkeley, CA) and *t*-test (StatView; Abacus Concepts). *P* values shown on figures and in Tables 1 and 2 were obtained by two-tailed *t*-test of differences between means. We further evaluated the within group treatment effects on cholesterol (Fig. 7, shown later) using the paired, two-tailed *t*-test. Mean ± SD values are recorded in tables; mean ± SEM values are plotted on figures.

RESULTS

The intervention for all subjects during weeks 4–8 phase was the AHA diet. We first examined the subjects' response to the AHA diet instructions. Although somewhat limited from a quantitative standpoint, our analysis (Table 1) reveals that the subjects significantly decreased their reported intakes of energy (23%, $P < 0.01$), carbohydrate (19%, $P < 0.05$), fat and saturated fatty acids (40%, $P < 0.03$; and 38%, $P < 0.01$, respectively), and cholesterol (33%, $P < 0.04$). Concomitant with these changes, there were modest, but significant, decreases (Table 2) in cholesterol (5%, $P < 0.03$), the estimate (9) of low density lipoprotein (LDL) cholesterol (8%, $P < 0.05$), and ApoB (9%, $P < 0.01$). All subjects were continued on the AHA diet during weeks 8–12 phase of the study. Our analysis revealed no significant differences between the subjects reported intakes at weeks 4–8 and weeks 8–12. This design enabled us to evaluate the impact of an eight-week exposure to the AHA dietary regimen on blood parameters of 16 subjects (Placebo, Figs. 1–6). Our analyses of differences

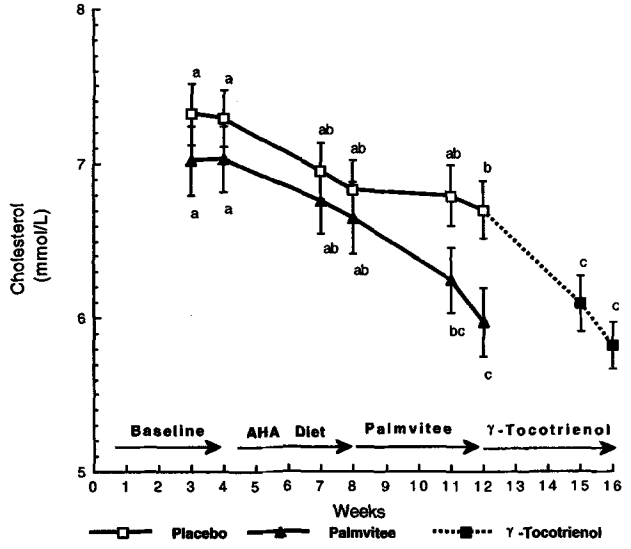


FIG. 1. Time-dependent impacts of American Heart Association Step 1 Diet (AHA) dietary regimen, Palmvitee (PORIM, Kuala Lumpur, Malaysia) and γ-Tocotrienol on serum cholesterol levels of hypercholesterolemic adult subjects. Differences between values on each plotted line which do not not share a letter are significant ($P < 0.05$).

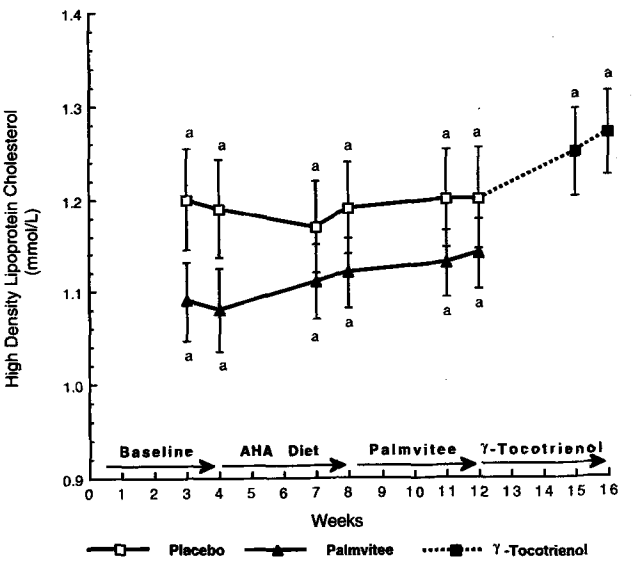


FIG. 3. Time-dependent impacts of AHA dietary regimen, Palmvitee, and γ-Tocotrienol on high density lipoprotein cholesterol levels of hypercholesterolemic adult subjects. Differences between values on each plotted line were not significant. Abbreviation and source as in Figure 1.

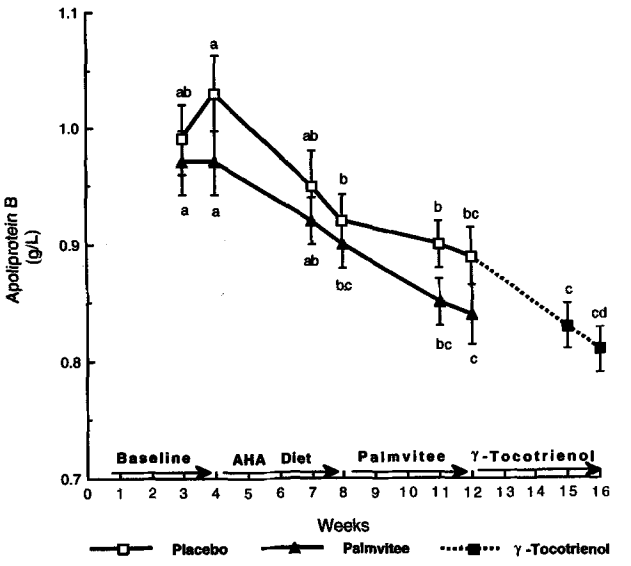


FIG. 2. Time-dependent impacts of AHA dietary regimen, Palmvitee, and γ-Tocotrienol on serum apolipoprotein B levels of hypercholesterolemic adult subjects. Differences between values on each plotted line which do not not share a letter are significant ($P < 0.05$). Abbreviation and company source as in Figure 1.

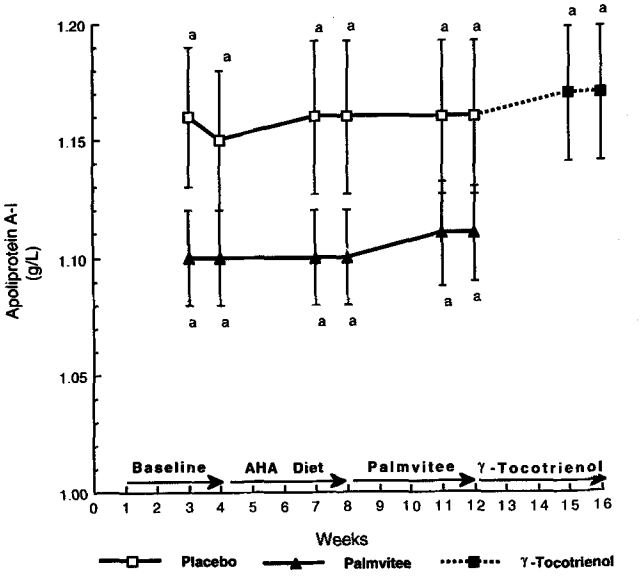


FIG. 4. Time-dependent impacts of AHA dietary regimen, Palmvitee, and γ-Tocotrienol on serum apolipoprotein A-I levels of hypercholesterolemic adult subjects. Differences between values on each plotted line were not significant. Abbreviation and company source as in Figure 1.

between Baseline (week four) and week 12 values reveal significant decreases in cholesterol (8%, $P < 0.05$, Fig. 1), LDL cholesterol (11%, $P < 0.05$), ApoB (13%, $P < 0.05$, Fig. 2), and glucose (9%, $P < 0.05$, Fig. 6). HDL cholesterol (Fig. 3), ApoA-I (Fig. 4), and triglyceride (Fig. 5) levels were not influenced by the dietary regimen. It should be noted that these subjects received 1.2 g corn oil in capsule form during weeks 8–12. In summary, the AHA diet proved to be effective in lowering the total cholesterol, LDL cholesterol (calculated, data not shown), ApoB, and glucose levels of this group of hypercholesterolemic subjects.

The cholesterol levels of the aforementioned 16 subjects decreased by 5 and 3% during the successive four-week exposures to the AHA dietary regimen. The cholesterol level of the remaining 20 subjects decreased by 5% during the first four-week exposure to the dietary regimen. These subjects re-

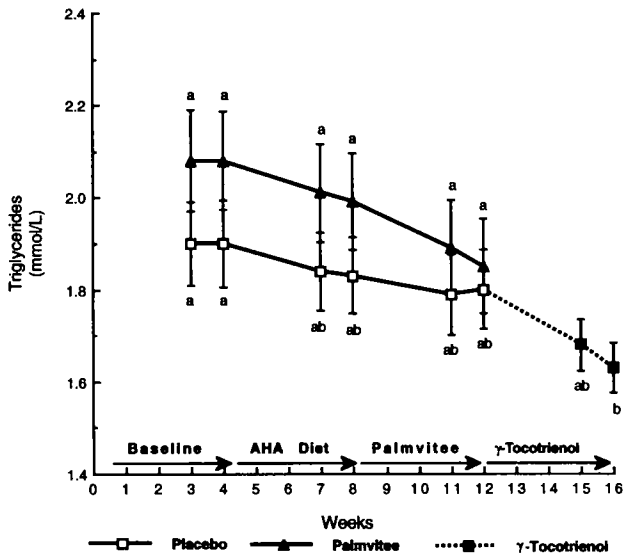


FIG. 5. Time-dependent impacts of AHA dietary regimen, Palmvitee, and γ-Tocotrienol on serum triglyceride levels of hypercholesterolemic adult subjects. Differences between values on each plotted line which do not share a letter are significant ($P < 0.05$). Abbreviation and company source as in Figure 1.

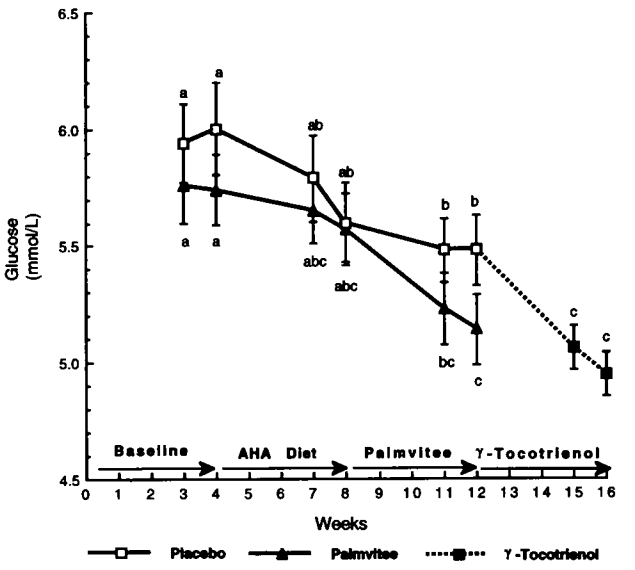


FIG. 6. Time-dependent impacts of AHA dietary regimen, Palmvitee, and γ-Tocotrienol on serum glucose levels of hypercholesterolemic adult subjects. Differences between values on each plotted line which do not share a letter are significant ($P < 0.05$). Abbreviation and company source as in Figure 1.

ceived Palmvitee, a tocotrienol-rich fraction of palm oil providing 40 mg α-tocopherol, 48 mg α-tocotrienol, 112 mg γ-tocotrienol, 60 mg δ-tocotrienol, and 1.14 g palm olein (0.6 g oleic acid, 0.15 g linoleic acid, and 0.4 g palmitic acid) during the second four-week exposure to the AHA diet. This combined regimen led to significant decreases in cholesterol (10%, $P < 0.05$), LDL cholesterol (13%, $P < 0.05$, calculated data not shown), and ApoB (7%, $P < 0.05$) levels during this four-week period (Figs. 1, 2). HDL cholesterol, ApoA-I, and triglyceride levels (Figs. 3–5) were not influenced by the combined regimen. Blood glucose at week 12 was 15% ($P < 0.05$) lower than the Baseline (week four) value (Fig. 6). In summary, the incremental response to Palmvitee likely accounted for 7% of the 10% decrease in cholesterol and associated decreases in LDL cholesterol and ApoB levels during this phase.

The tocol mixture in the Palmvitee supplement was comprised of 15% α-tocopherol and 85% tocotrienols. It has previously been noted that α-tocopherol attenuates the cholesterol-suppressive actions of the tocotrienols (5,6). Therefore, it was of interest to evaluate the impact of a tocopherol-free preparation of γ-tocotrienol on the cholesterol levels of the 16 Placebo subjects during a terminal four-week phase of the study. As shown on Figure 1, the cholesterol level of these subjects had plateaued during their second four-week exposure to AHA dietary regimen. The γ-tocotrienol supplement effected a significant 13% decrease in cholesterol (Fig. 1), with concomitant decreases in ApoB (Fig. 2) and LDL cholesterol (calculated, not shown) levels. The significant decrease in blood glucose during this period reflects that previously noted on the exposure of the other subjects to Palmvitee (Fig. 6). HDL cholesterol and ApoA-I levels remained stable (Figs. 3, 4). Finally, we note that the general decrease in triglycerides achieved statistical significance (Fig. 5). We postulate that this decrease reflects the long-range impact of the AHA dietary regimen.

We further evaluated the treatment effects on cholesterol levels using the paired *t*-test. We first calculated the difference between week 8 and week 4, week 12 and week 8, and week 16 and week 12 values for each individual (Scheme 1). The AHA diet-mediated decrease in cholesterol for the Placebo group during the first four-week exposure (–0.40 ± 0.07 mmol/L) was significantly greater than that during the second four-week exposure (–0.20 ± 0.03 mmol/L, $P < 0.005$). The decrease in cholesterol for the Palmvitee group during the four-week exposure to the AHA diet (–0.38 ± 0.05 mmol/L) equaled that observed in the Placebo group. This decrease, however, was significantly less than that observed when the subjects received Palmvitee (–0.68 ± 0.12, $P < 0.050$). This impact of tocotrienols is confirmed in the final comparison showing that the decrease in cholesterol during the γ-tocotrienol phase of the experiment (–0.87 ± 0.10 mmol/L) is significantly greater than that which occurred during the second four-week exposure to the AHA dietary regimen ($P < 0.001$).

Serum TxB_2 concentrations decreased significantly ($P < 0.05$) during the Palmvitee and γ-Tocotrienol phases of the study (Fig. 7).

DISCUSSION

The present study was designed to avoid the previously noted confounding factors which we believe underlie the failure of others (4) to demonstrate the cholesterol-suppressive action

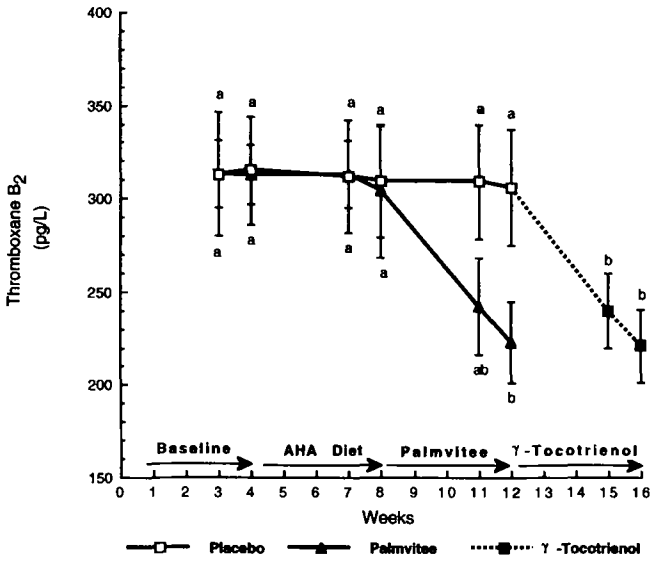


FIG. 7. Time-dependent impacts of AHA dietary regimen, Palmvitee, and γ-Tocotrienol on thromboxane B_2 concentrations in serum of hypercholesterolemic adult subjects. Differences between values on each plotted line which do not not share a letter are significant ($P < 0.05$). Abbreviation and company source as in Figure 1.

of the tocotrienols. Hypercholesterolemic subjects were first conditioned for four weeks to a dietary regimen patterned after the AHA Diet. During that acclimation period, energy intake, estimated from spontaneous diet recalls, decreased by 22%; the proportion of energy supplied by fat decreased from 34 to 30% and that provided by saturated fatty acids, from 12 to 9%. Cholesterol intake decreased from 123 to 106 mg/1000 kcal (Table 1). Four weeks' acclimation to the AHA Diet resulted in significant decreases in cholesterol (5%) and LDL cholesterol (8%) levels. The group of subjects continuing on the AHA Diet for another four weeks experienced an additional 2% decrease in these parameters. Cholesterol (Fig. 1) and LDL cholesterol after eight weeks' exposure to the AHA Diet were decreased by 7 and 10%, respectively.

As previously noted, this study was designed to segregate the impact of the dietary regimen from that of the tocotrienol supplements. Our analysis revealed no significant changes in dietary intakes during the Palmvitee phase of the study. Fat and saturated fatty acids provided 28 and 9%, respectively, of energy intake; cholesterol intake increased from 106 to 134 mg/1000 kcal. The cholesterol-suppressive action of Palmvitee achieved significance at four weeks (Fig. 1); this change is supported by concomitant and significant Palmvitee-induced changes in LDL cholesterol (not shown) and ApoB (Fig. 2).

The Palmvitee capsules provided 220 mg tocotrienols and 40 mg α-tocopherol. The *in vivo* cholesterol-lowering action of the tocotrienols is attenuated by α-tocopherol (5,6), and the tocotrienols differ significantly in their capacity to suppress cholesterol synthesis; γ-tocotrienol and δ-tocotrienol are approximately equipotent, whereas α-tocotrienol has a lower potency (10). These considerations led us to evaluate the cholesterol-suppressive action of a tocotrienol preparation free of α-tocopherol. This preparation provided 200 mg γ-tocotrienol, a quantity calculated to have the cholesterol-suppressive potency of the 220 mg mixture of tocotrienols in the Palmvitee. As shown on Figure 1, the decrease in serum cholesterol mediated by the tocopherol-free preparation reached significance at three weeks.

The significant tocotrienol-mediated decrease in serum cholesterol (Fig. 1) and ApoB (Fig. 2) (and LDL cholesterol) levels of hypercholesterolemic subjects were accomplished in the absence of a treatment-mediated impact on levels of HDL cholesterol (Fig. 3) and ApoA-I (Fig. 4). This response is at odds with that reported by by Wahlqvist *et al.* (4). They conducted a 26-week (six weeks run-in and 20 weeks experimental), rising-dose study with a Palmvitee preparation (23% α-tocotrienol, 31% γ-tocotrienol, 16% δ-tocotrienol, and 30% α-tocopherol). After the run-in period (unspecified dietary fat modification), 52 hypercholesterolemic subjects were randomly assigned to placebo (n = 26, 6.95 ± 1.58 mmol cholesterol/L) and rising-dose treatment (n = 26, 7.51 ± 1.94 mmol cholesterol/L) groups. Interpretation of the results of this study presents several problems, including that due to having a different number of subjects per group (17 to 44). The authors report that only four of the 17 subjects receiving Palmvitee from week 0 to week 16 experienced a fall in serum cholesterol. Tan *et al.* (3) reported that 14 of 15 hypercholesterolemic subjects receiving a similar preparation of Palmvitee (30% α-tocopherol) experienced a greater than 5% decrease in cholesterol. In our earlier study (2), cholesterol levels of 12–15 hypercholesterolemic subjects receiving a preparation of Palmvitee (20% α-tocopherol) decreased. As previously noted, the failure of some subjects to respond may indicate that the biological action of tocotrienols may be more complex than simply inhibiting HMG–CoA reductase.

We found significant tocotrienol-mediated decreases in serum TxB_2 (Fig. 7). This decrease, we postulate, is consistent with the finding that the tocotrienols in membrane redox systems have a major, but little appreciated, antioxidant capacity (11) that would be detected in this capacity-related *ex vivo* index of platelet cyclooxygenase activity. Whether this might reflect an additional cardiovascular benefit *in vivo* remains to be determined.

ACKNOWLEDGMENTS

We thank Norhaya Hashim (PORIM) and Lei He (Department of Nutritional Sciences, University of Wisconsin) for their technical help. This study was supported in part by a grant from the Palm Oil Research Institute of Malaysia (PORIM) and the college of Agricultural and Life Sciences, University of Wisconsin.

REFERENCES

1. Parker, R.A., Pearce, B.C., Clark, R.W., Gordon, D.A., and Wright, J.J.K. (1993) Tocotrienols Regulate Cholesterol Production in Mammalian Cells by Post-Transcriptional Suppression of 3-Hydroxy-3-Methylglutaryl–Coenzyme A Reductase, *J Biol. Chem. 268*, 11230–11238.

2. Qureshi, A.A., Qureshi, N., Wright, J.J.K., Shen, Z., Kramer, G., Gapor, A., Chong, Y.H., DeWitt, G., Ong, A.S.H., Peterson, D.M., and Bradlow, B.A. (1991) Lowering of Serum Cholesterol in Hypercholesterolemic Humans by Tocotrienols (Palmvitee), *Am. J. Clin. Nutr. 53,* 1021S–1026S.
3. Tan, D.T., Khor, H.T., Low, W.H.S., Ali, A., and Gapor, A. (1991) Effect of a Palm-Oil-Vitamin E Concentrate on the Serum and Lipoprotein Lipids of Humans, *Am. J. Clin. Nutr. 53,* 1027S–1031S.
4. Wahlqvist, M.L., Krivokuca-Bogetic, Z., Lo, C.H., Hage, B., Smith, R., and Lukito, W. (1992) Differential Serum Responses to Tocopherols and Tocotrienols During Vitamin E Supplementation in Hypercholesterolaemic Individuals Without Change in Coronary Risk Factors, *Nutr. Res. 12,* S181–S201.
5. Qureshi, A.A., Nor, R.M., Gapor, A., Peterson, D.M., Pearce, B.C., and Elson, C.E., α-Tocopherol Attenuates the Impact of γ-Tocotrienol on 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity, *J. Nutr., in* press
6. Qureshi, A.A., Burger, W.C., Peterson, D.M., and Elson, C.E (1986) The Structure of an Inhibitor of Cholesterol Biosynthesis Isolated from Barley, *J. Biol. Chem. 261,* 10544–10550.
7. Anonymous (1982) Rationale of the Diet-Heart Statement of the American Heart Association. Report of the AHA Nutrition Committee (Review), *Arteriosclerosis 2,* 177–191.
8. Anonymous (1988) Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. The Expert Panel, *Arch. Intern. Med. 148,* 36–69.
9. Friedwald, W.T., Levy, R.I., and Fredrickson, D.S. (1972) Estimation of the Concentration of Low Density Lipoprotein Cholesterol in Plasma Without Use of Preparative Ultracentrifuge, *Clin. Chem.18,* 499–502.
10. Pearce, B.C., Parker, R.A., Deason, M.E., Qureshi, A.A., and Wright, J.J.K. (1992) Hypocholesterolemic Activity of Synthetic and Natural Tocotrienols, *J. Med. Chem. 35,* 3595–3606.
11. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) Free Radical Recycling and Intramembrane Mobility in the Antioxidant Properties of alpha-Tocopherol, and alpha-Tocotrienol, *Free Radic. Biol. Med. 10,* 263–275.

[Received January 12, 1994, and in final revised form October 13, 1995; Revision accepted October 18, 1995]

Antioxidant Effects of Tocotrienols in Patients with Hyperlipidemia and Carotid Stenosis[1]

A.C. Tomeo[a], M. Geller[b], T.R. Watkins[a], A. Gapor[c], and M.L. Bierenbaum[a,*]

[a]Kenneth L. Jordan Research Group, Montclair, New Jersey 07042, [b]Elmhurst Medical Center, Queens, New York, [c]Palm Oil Research Institute of Malaysia, Kuala Lumpur, Malaysia

ABSTRACT: Antioxidants may have a role in the prevention of atherosclerosis. In the present trial, we investigated the antioxidant properties of Palm Vitee, a γ-tocotrienol-, and α-tocopherol enriched fraction of palm oil, in patients with carotid atherosclerosis. Serum lipids, fatty acid peroxides, platelet aggregation, and carotid artery stenosis were measured over an 18-month period in fifty patients with cerebrovascular disease. Change in stenosis was measured with duplex ultrasonography. Ultrasound scans were done at six months, twelve months, and yearly thereafter. Bilateral duplex ultrasonography revealed apparent carotid atherosclerotic regression in seven and progression in two of the 25 tocotrienol patients, while none of the control group exhibited regression and ten of 25 showed progression ($P < 0.002$). Serum thiobarbituric acid reactive substances, an *ex vivo* indicator of maximal platelet peroxidation, decreased in the treatment group from 1.08 ± 0.70 to 0.80 ± 0.55 μM/L ($P < 0.05$) after 12 mon, and in the placebo group, they increased nonsignificantly from 0.99 ± 0.80 to 1.26 ± 0.54 μM/L. Both tocotrienol and placebo groups displayed significantly attenuated collagen-induced platelet aggregation responses ($P < 0.05$) as compared with entry values. Serum total cholesterol, low density lipoprotein cholesterol, and triglyceride values remained unchanged in both groups, as did the plasma high density lipoprotein cholesterol values. These findings suggest that antioxidants, such as tocotrienols, may influence the course of carotid atherosclerosis.

Lipids 30, 1179–1183 (1995).

In recent years, evidence has linked processes involving oxygen-derived free radicals with the initiation and propagation of atherosclerosis. In particular, the oxidative modification of low density lipoproteins (LDL), with its ensuing sequelae of cytotoxic, thrombogenic, and chemotactic events, has emerged as a key step in promoting atherosclerosis (1–3). Antioxidants, especially vitamin E, have received considerable attention as potential antiatherogenic agents for over 40 years. A number of recent studies have demonstrated the ability of antioxidants to prevent *ex vivo* and *in vitro* LDL oxidation, and to potentially be able to ameliorate the development of atherosclerotic lesions (4–6). Epidemiological studies have linked the dietary intake of vitamin E and other antioxidants with a reduced risk of coronary heart disease (7,8), ischemic heart disease (9), ischemic stroke (10), coronary and total mortality (11), and with a decrease in carotid artery wall thickness (12). As the vitamin-rich distillate of palm oil [Palm Vitee; Palm Oil Research Institute of Malaysia (PORIM), Kuala Lumpur, Mayalsia] is enriched with several antioxidant members of the vitamin E family (tocopherols and tocotrienols), it seemed worthwhile to investigate its antioxidant effects in patients with cerebrovascular disease.

EXPERIMENTAL PROCEDURES

The study cohort consisted of 50 subjects (23 males and 27 females), ranging in ages 49–83 yr, with carotid artery atherosclerosis as determined by duplex carotid ultrasonography. Patient numbers 7 and 13, one from each group, dropped out and were excluded. They were replaced by patient numbers 51 and 52 in the analysis. Eligibility criteria included subjects less than 85 years of age who had either had a hemispheric transient ischemic attack, monocular blindness for less than 24 h, or a nondisabling hemispheric stroke within the previous year. Subjects had carotid artery stenosis ranging from 15 to 79%. Subjects having stenosis beyond 79% were referred to surgery. Forty-four percent of placebo and 60% of test subjects had more than 49% stenosis of the carotid artery. All patients continued to receive their current medical care, including antihypertensive, antidiabetic, and antiplatelet (usually aspirin) treatment, except that 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors were discontinued.

At baseline, patients underwent standardized history, physical, and neurological examinations, an assessment of functional status, standardized laboratory tests, 12-lead electrocardiography, a chest X-ray, and duplex carotid ultrasonography. A synopsis of subject characteristics appear at baseline showing their similarity [Table 1; collagen-induced aggregation (Ω) was 17.3 for the antioxidant group, 15.5 for the placebo group; thrombin ATP release (μM/L) was 1.21 ± 1.1 and 1.02 ± 0.39 for the antioxidant and placebo groups, respectively].

Ultrasonography of the carotid arteries was done at baseline and repeated at six and twelve months, and annually thereafter. Degree of stenosis was graded as: 0–15%; 16–49%;

[1]Based on a paper presented at the PORIM International Palm Oil Congress (PIPOC) held in Kuala Lumpur, Malaysia, September 1993.

*To whom correspondence should be addressed at 48 Plymouth St., Montclair, NJ 07042.

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; TBARS, thiobarbituric acid reactive substances.

Copyright © 1995 by AOCS Press

TABLE 1
Comparison of Patient Characteristics at Study Entry by Treatment Group[a]

Patient characteristic	Antioxidant (n = 25)	Placebo (n = 25)	P^b
Age (yr)	66.4	66.7	n.s.
Gender (F/M)	13/12	14/11	n.s.
Antiplatelet (aspirin) use (no.)	15	11	n.s.
No anticoagulant use (no.)	21	24	n.s.
Hypertensives (no.)	9	14	n.s.
Diabetics (no.)	5	2	n.s.
Abnormal EKG[b] (no.)	16	15	n.s.
Abnormal chest X-ray[b] (no.)	7	12	n.s.
Cigarette use (no.)	5	4	n.s.
Height, inches (m)	64.8 ± 4.2 (1.65 ± 0.1)	64.4 ± 3.5 (1.63 ± 0.1)	n.s.
Weight, lbs (kg)	152.8 ± 39.5 (69.4 ± 17.9)	150.8 ± 26.9 (68.6 ± 12.2)	n.s.
Systolic Blood pressure (mm Hg)	138.6 ± 14.4	147.8 ± 20.0	
Diastolic blood pressure (mm Hg)	82.4 ± 9.7	81.2 ± 9.6	n.s.
No. > 49% carotid stenosis	15	11	n.s.

[a]Data expressed as means ± SD; n.s., not significant.
[b]Abnormal for cardiovascular pathology.

50–79%, and 80–99%. Ultrasound duplex measurements were done with an Acuson 128 XP ultrasonograph (Acuson Co., Mountain View, CA) equipped with a 5 or 7 MHz transducer. The transducer aperture was 38 mm. Subjects were examined in the supine position. Change from one category to the next was reckoned regression or progression; a change of two categories was considered marked regression or progression. All ultrasound studies were done by the same person, who was unaware of treatment assignments and results of earlier measurements. The artery of neurological involvement was used for study, in an attempt to more easily correlate the pathological and clinical changes.

Using a table of random numbers, patients were assigned to receive 300 mg capsules containing either 16 mg α-tocopherol, 40 mg γ- and α-tocotrienols, and 240 mg palm superolein, (Palm Vitee), or placebo (300 mg palm superolein). Palm superolein is a mixture of triglycerides with the predominant fatty acids being oleic, palmitic, and linoleic (6:4:1.51 ratio). Capsules containing placebo or palm oil fraction were provided by PORIM. Initial dosage of the palm oil antioxidants or placebo was four capsules daily. At the three- and six-month follow-up visits, the dosage was increased to five and six capsules daily, respectively, in an attempt to lower serum lipids. The dosage then remained at six capsules/day (240 mg tocotrienol) for the remainder of the study. Patients were blinded throughout the trial; none in either group reported side effects. Investigators also were blinded about treatment groups, except for the one who distributed the capsules.

During the baseline, 3-, 6-, 9-, 12-, and 18-month visits, venous blood was drawn after overnight fasting into EDTA and Vacutainer SST tubes (Becton Dickinson Co., Rutherford, NJ) with a sterile activator, stored at ambient temperature for five minutes, spun at ambient temperature at 3400 rpm, and either analyzed immediately or stored frozen overnight before analysis. Though samples were identified by name during analysis, the analyses were done in a laboratory separate from the outpatient clinic. A lipid profile was measured in each subject to control for dietary changes or medicinal usage compliance which might have affected stenosis. It included serum total cholesterol, LDL cholesterol (by difference) (13), and triglycerides for each visit using Reflotron methodology, which uses cholesterol esterase, oxidase, horseradish peroxidase, and 3,3′,5,5′-tetramethylbenzidine dye, which is quantitated by reflectance (Boehringer-Mannheim, Indianapolis, IN) and high density lipoprotein (HDL) cholesterol using the same chemistry with EDTA-treated plasma. Triglycerides were quantitated by reaction with an esterase, glycerol kinase, glycerol phosphate oxidase, and peroxidase in the presence of a dye [4-(4-dimethylaminophenyl)-5-methyl-2-(4-hydroxy-3,5-dimethyloxyphenol)-imidazole-dihydrochloride]. All analyses were done under amber light to reduce photooxidation. Fatty acid hydroperoxides were assayed according to Ohishi *et al.* (14). Cumene hydroperoxide was used as the standard. The serum level of thiobarbituric acid reactive substances (TBARS) was measured to quantitate malondialdehyde equivalent materials by the method of Mihara *et al.* (15). Serum levels of α-tocopherol were measured during baseline and at the 18-month visits by a high-performance liquid chromatography method of Bieri *et al.* (16) to monitor compliance. This method was not able to separate and detect measurable tocotrienols. Platelet aggregation in whole blood was measured with an impedance aggregometer (Chronolog Corp., Havertown, PA), using thrombin and collagen as agonists to initiate aggregation (17), measuring only the primary wave of aggregation after the reaction had proceeded for six minutes. These measurements were made on a background of aspirin, as all subjects were taking aspirin during the trial.

Subject compliance was monitored by pill counts and measurement of serum vitamin levels, as indicated with each data panel. Pill counts showed 95% compliance.

Statistics were performed using Student's *t*-test, the paired *t*-test, or the Mann-Whitney test. Available 3-, 6-, 9-, 12-, or 18-month data are presented.

RESULTS

Those patients being supplemented with tocopherol and tocotrienols for 18 mon approximately doubled their serum α-tocopherol levels compared to baseline (Table 2). Serum levels of tocotrienol were below the detection limits of our high-performance liquid chromatography system; hence, no tocotrienol data are presented.

The percentage change of carotid stenosis in patients by category differed in the groups receiving palm oil antioxidants and placebo after 12 mon of treatment. The data indicated (Table 3) that six (24%) of the subjects supplemented with antioxidants for 12 mon moved one category to a lesser apparent degree of stenosis; one (4%) improved two categories, suggesting marked regression. Only two from this group (8%) exhibited progression of carotid atherosclerosis. The remaining 64% manifested no change in disease state. In contrast, 24% of the placebo group appeared to progress, and 16% markedly progressed in carotid stenosis ($P < 0.002$; Mann-Whitney), while 50% of doppler studies from the placebo group remained unchanged. No one receiving the placebo showed carotid plaque regression. These data were little changed from the 6-month findings.

Data of the effect of the palm oil antioxidants vs. placebo in lowering serum lipid oxidation products are shown (in Table 2). Patients receiving antioxidants attenuated their generations of TBARS after 12 mon, with values decreasing from (mean ± SD) 1.08 ± 070 to 0.80 ± 0.55 μM/L ($P < 0.05$); whereas placebo subjects' values rose from 0.99 ± 0.80 to 1.26 ± 0.54 μM/L (nonsignificant). At baseline, a difference was seen in hydroperoxide levels, possibly attributed to cigarette smoking (Table 1). Although not significant, the fatty acid hydroperoxides increased in both groups, rising from 2.2 ± 1.26 at baseline to 3.6 ± 9.4 μM/L after 12 mon in the test group and 4.5 ± 6.4 to 3.6 ± 7.5 μM/L in the placebo group.

After 12 mon, both antioxidant supplement (16.9 ± 12.0 Ω to 12.0 ± 9.0 Ω; $P < 0.05$) and placebo (17.7 ± 12.9 Ω to 12.0 ± 7.8 Ω; $P < 0.02$) were associated with attenuation of collagen-induced platelet aggregation when compared with baseline. The platelet ATP response to thrombin did not change in the placebo group (1.02 ± 0.39 to 1.04 ± 0.73), but changed from 0.99 ± 0.46 to 1.24 ± 0.76 (nonsignificant) in the antioxidant group. The improvement seen in platelet aggregation may have resulted from improved compliance with respect to prescribed medication as a result of participation in the study. Because both groups used aspirin, and aspirin usage was monitored during the study, the decline seen in platelet aggregation was attributed to this cyclooxygenase inhibitor.

Treatment with the palm oil fraction providing 240 mg tocotrienols daily for 18 mon had no measurable effect on serum lipids (Table 4). Compared to baseline, serum total, LDL and HDL cholesterol, and triglyceride values remained virtually unchanged (nonsignificant) in test and in control groups over the observation periods.

DISCUSSION

The purpose of the present clinical study was to test the effect of tocotrienols in patients with carotid atherosclerosis. After extending the duration of the tocotrienol supplementation study to 18 mon, although we found no measurable improvement in blood lipids, we observed an apparently improved blood flow in the carotid arteries of six patients out of 25. Over a one-year period, in a population of hypercholesterolemic Eastern Finnish men, an inverse correlation was observed between plasma antioxidant levels (vitamin E, β-carotene, selenium) and the increase in carotid intima-media thickness (18). Similarly, in both men and women, an inverse

TABLE 2
Serum TBARS, LOPS, and α-Tocopherol Values and Platelet Responses of Subjects Given Tocotrienols and Tocopherol or Placebo for 18 Mon[a]

	Placebo					
Analyte	Baseline	3 mon	6 mon	9 mon	12 mon	18 mon
Collagen (Ω)	17.7 ± 12.7	12.6 ± 8.3	14.9 ± 10.1	15.4 ± 8.4	12.0 ± 7.8[c]	14.1 ± 7.0
ATP (μM/L)[b]	1.02 ± 0.39	0.97 ± 0.49	0.99 ± 0.69	1.00 ± 0.64	1.04 ± 0.73	0.91 ± 0.42
TBARS (μM/L)	0.99 ± 0.80	1.02 ± 0.73	1.01 ± 0.71	1.21 ± 0.73	1.26 ± 0.54[d]	1.42 ± 0.69
LOPS (μM/L)	2.19 ± 1.26	1.58 ± 1.0	2.1 ± 1.6	6.7 ± 15.7	3.6 ± 9.4	2.8 ± 3.5
Vitamin E (μM/L)	19.0 ± 5.8	—	—	—	—	13.9 ± 7.7[e]
	Palm oil fraction (tocotrienols)					
Collagen (Ω)	16.9 ± 12.0	14.6 ± 12.9	9.52 ± 8.28	15.6 ± 11.2	12.0 ± 9.0[c]	13.2 ± 10.9
ATP (μM/L)[b]	0.99 ± 0.46	1.02 ± 0.42	0.92 ± 0.47	0.92 ± 0.47	1.24 ± 0.76	1.24 ± 0.46
TBARS (μM/L)	1.08 ± 0.70	0.66 ± 0.52	0.68 ± 0.55	0.68 ± 0.56	0.80 ± 0.55[d]	1.24 ± 1.11
LOPS (μM/L)	4.5 ± 6.4	1.58 ± 1.0	4.52 ± 6.4	4.1 ± 5.7	3.6 ± 7.5	2.6 ± 2.8
Vitamin E (μM/L)	19.7 ± 7.7	—	—	—	—	37.4 ± 15.6[e]

[a]Data expressed as mean ± SD. TBARS, thiobarbituric acid reactive substances; LOPS, fatty acid hydroperoxides.
[b]Thrombin agonist challenge, 1 NIH unit/mL.
[c]$P < 0.05$ vs. respective baseline value.
[d]$P < 0.05$ vs. baseline and palm oil fraction (tocotrienols) vs. placebo.
[e]$P < 0.01$ palm oil fraction (tocotrienols) vs. baseline and Palm Vitee 18 mon vs. placebo 18 mon.

TABLE 3
Comparison of Change in Carotid Stenosis in Groups Receiving Tocotrienols or Placebo for Six and Twelve Months[a]

	Antioxidant	Placebo
Six months		
MR	1	0
R	5	0
NC	18	20
P	1	2
MP	0	3
Total number	25	25
Twelve months		
MR	1	0
R	6	0
NC	16	15
P	2	6
MP	0	4
Total number	25	25

[a]Data expressed as number of subjects per category. MR, marked regression; R, regression; NC, no change; P, progression; MP, marked progression.

relationship was found between dietary antioxidant intake (α-tocopherol, ascorbic acid, β-carotene) and carotid artery wall thickness (12).

To date, the precise mechanism(s) responsible for plaque regression have not been fully elucidated and appear to be complex and multifactorial. Earlier work from this laboratory had documented the effective inhibition of platelet aggregation by antioxidants in hyperlipemic patients (19). After taking into account that one measure of the thrombogenicity of the blood was equally attenuated in both groups, and that the serum lipid profiles remained stable, it appears that the generation of serum TBARS data may provide a clue about the mode of action of tocotrienol. Elegant studies aptly support the theory that antioxidants curtail the levels of blood lipid peroxides, and blunt the formation of atherosclerotic lesions. Esterbauer *et al.* (4) demonstrated vitamin E to be very effective in preventing *in vitro* oxidation of LDL, and Carew *et al.* (5) showed that the antioxidant probucol inhibited the progression of atherosclerotic lesions in LDL receptor-deficient rabbits.

Throughout this trial, we have observed no change in serum lipid levels. To the contrary, Qureshi *et al.* (20,21) have reported a reduction in both serum total and LDL cholesterol in human hyperlipidemics and pigs supplemented with tocotrienol. Wahlquist *et al.* (22), however, observed a differential response to tocopherol and tocotrienol supplementation in human subjects without any change in serum lipids. In preliminary trials with a rat model, we had observed a hypolipidemic effect with γ-tocotrienol (23); in this regard, our results in rats did not predict the outcome in patients on uncontrolled diets who were taking a variety of medications. From the present data, it appears that a decrease in serum cholesterol was not a prerequisite in the six patients suggested to have improved carotid atherosclerosis. This impression is in contrast to coronary atherosclerosis, as we reported earlier (24). Cholesterol is, indeed, a poor predictor of carotid, compared to coronary, plaque thickness. Miettenen *et al.* (25) have reported that although no significant decrease in coronary risk was measured when clofibrate was given with probucol, no stroke cases were observed in this 1200-member study. Mao *et al.* (6) demonstrated that the attenuation of atherosclerotic lesions in Watanabe rabbits by a probucol analogue was not contingent on a concomitant reduction in serum cholesterol levels. Similarly, probucol reduced the incidence of atherosclerotic lesions in a group of Japanese hypercholesterolemic quail without altering their plasma total or lipoprotein cholesterol levels (26). These investigators attributed this effect to probucol's antioxidant properties. However, in view of the recent findings that probucol results in lowered HDL_{2b} levels in hypercholesterolemics (27), probucol use in atherosclerosis has been viewed with caution, and its manufacturer has recently indicated that this drug will be withdrawn from the U.S. market.

Our results highlight the need to investigate further roles of antioxidants such as tocotrienols in preventing, and possibly reversing, the natural course of carotid atherosclerosis.

Finally, two recent meta-analyses of randomized, controlled trials (28,29) found that lowering serum cholesterol through modified diets or medications did not reduce stroke mortality or morbidity in middle-aged men.

TABLE 4
Comparison of Response of Study Groups to 18-Month Antioxidant Supplementation on Serum Cholesterol Fractions and Triglycerides[a]

Serum moiety	Antioxidants						
(mM/L)	Baseline	3	6	9	12	18	*P*
Cholesterol	6.05 ± 0.03	6.01 ± 1.40	6.15 ± 1.57	6.10 ± 1.31	6.17 ± 1.63	6.20 ± 1.47	n.s.
LDL-chol.	4.24 ± 0.03	4.27 ± 1.36	4.39 ± 1.51	4.25 ± 1.08	4.39 ± 1.44	4.42 ± 1.19	n.s.
HDL-chol.	0.98 ± 0.01	1.01 ± 0.31	0.92 ± 0.22	0.98 ± 0.25	1.00 ± 0.34	1.08 ± 0.35	n.s.
Triglyceride[b]	5.67 ± 3.84	5.03 ± 3.09	7.08 ± 5.99	5.96 ± 5.09	5.45 ± 3.28	4.53 ± 2.48	n.s.
	Placebo						
Cholesterol	5.90 ± 0.16	5.86 ± 1.07	5.68 ± 1.39	6.03 ± 1.35	5.67 ± 1.32	5.92 ± 0.10	n.s.
LDL-chol.	4.19 ± 0.14	4.15 ± 1.14	4.00 ± 1.33	4.14 ± 1.52	4.01 ± 1.33	4.21 ± 0.88	n.s.
HDL-chol.	1.10 ± 0.01	1.12 ± 0.34	1.10 ± 0.27	1.10 ± 0.31	1.15 ± 0.32	1.04 ± 0.24	n.s.
Triglyceride[b]	4.71 ± 4.10	3.70 ± 1.53	4.92 ± 4.52	5.20 ± 5.16	3.85 ± 1.78	4.20 ± 1.76	n.s.

[a]LDL-chol., low density lipoprotein cholesterol; HDL-chol., high density lipoprotein cholesterol. Data expressed as mean ± SD; n.s., not significant.
[b]Average molecular weight 914 daltons.

ACKNOWLEDGMENT

The K.L. Jordan Research Group gratefully acknowledges the support in these studies of PORIM (Kuala Lumpur, Malaysia).

REFERENCES

1. Salonen, J.T., Yla-Herttuala, S., Yamamoto, R., Butler, S., Korpella, H., Salonen, R., Nyyssonen, K., Palinski, W., and Witztum, J.L. (1992) Autoantibody Against Oxidized LDL and Progression of Carotid Atherosclerosis, *Lancet 339*, 883–887.
2. Witztum, J.L., and Steinberg, D. (1991) Role of Oxidized Low Density Lipoprotein in Atherogenesis, *J. Clin. Invest. 88*, 1785–1792.
3. Steinberg, D., and Workshop Participants (1992) Antioxidants in the Prevention of Atherosclerosis, *Circulation 85*, 2338–2344.
4. Esterbauer, H., Dieber-Rotheneder, M., Striegel, G., and Waeg, G. (1991) Role of Vitamin E in Preventing the Oxidation of Low-Density Lipoprotein, *Am. J. Clin. Nutr. 3*, 314S–321S.
5. Carew, T.E., Schwenke, D.C., and Steinberg, D. (1987) Antiatherogenic Effect of Probucol Unrelated to Its Hypocholesterolemic Effect: Evidence That Antioxidants *in vivo* Can Selectively Inhibit Low Density Lipoprotein Degradation in Macrophage-Rich Fatty Streaks Slowing the Progression of Atherosclerosis in the WHHL Rabbit, Proc. *Natl. Acad. Sci. (USA) 84*, 7725–7729.
6. Mao, S.J.T., Yates, M.T., Parker, R.A., Chi, E.M., and Jackson, R.L. (1991) Attenuation of Atherosclerosis in a Modified Strain of Hypercholesterolemic Watanabe Rabbit with Use of a Probucol Analogue (MD29,311) That Does Not Lower Serum Cholesterol, *Arterioscl. and Thromb. 11*, 1266–1275.
7. Rimm, E.B., Stampfer, M.J., Ascherio, A., Giovanucci, E., Colditz, G.A., and Willett, W.C. (1993) Vitamin E Consumption and the Risk of Coronary Heart Disease in Men, *N. Engl. J. Med. 328*, 1450–1456.
8. Stampfer, M.J., Hennekens, C.H., Manson, J.E., Colditz, G.A., Rosner, B., and Willett, W.C. (1993) Vitamin E and Risk of Coronary Disease in Women (1993) *N. Eng. J. Med. 328*, 1444–1449.
9. Gey, K.F. (1995) Ten-Year Retrospective on the Antioxidant Hypothesis of Arteriosclerosis, *J. Nutr. Biochem. 6*, 206–236.
10. Manson, J.E., Stampfer, M.J., Willett, W.C., Colditz, G.A., Speizer, F.E., and Hennekens, C.H. (1995) Antioxidant Vitamin Consumption and Incidence of Stroke in Women, *New Engl. J. Med., 23,* November, in press.
11. Pandey, D., Shekelle, R., Tangney, C., and Stamler, J. (1995) Vitamin C and β-Carotene and Risk of Death in Middle-Aged Men in the Western Electric Study, *Am. J. Epidem.*, in press.
12. Kritchevsky, S.B., Shimakawa, T., Dennis, B., Eckfeldt, J., Carpenter, M., and Heiss, G. (1993) Dietary Antioxidants and Carotid Artery Wall Thickness: ARIC Study, *Circulation 87*, 3.
13. Delong, D.M., Delong, R., Wood, P.D., Lippel, K., and Rifkind, B.M. (1986) A Comparison of Methods for the Estimation of Plasma Low- and Very Low-Density Lipoprotein Cholesterol, *J. Am. Med. Assoc. 256*, 2372–2377.
14. Ohishi, N., Ohkawa, H., Miike, A., Tatano, T., and Yagi, K. (1985) A New Assay Method for Lipid Peroxides Using a Methylene Blue Derivative, *Biochem. Intl. 10*, 205–211.
15. Mihara, M., Uchiyama, M., and Fukuzawa, K. (1980) Thiobarbituric Acid Value on Fresh Homogenate of Rat as a Parameter of Lipid Peroxidation in Aging; CCl_4 Intoxication and Vitamin E Deficiency, *Biochem. Med. 23*, 302–311.
16. Bieri, J.G., Tolliver, T.J., and Catigniani, C.L. (1979) Simultaneous Determination of α-Tocopherol and Retinol in Plasma and Red Cells by High Pressure Liquid Chromatography, *Am. J. Clin. Nutri. 32*, 2143–2149.
17. Galvez, A., Badimon, L., Badimon, J., and Fuster, V. (1986) Electrical Aggregometry in Whole Blood from Human, Pig, and Rabbit, *Thromb. and Hem. 56*, 128–132.
18. Salonen, J.T., Nyssonen, K., Parviainen, M., Kantola, M., Kantola, M., Korpela, H., and Solonen, R. (1993) Low Plasma β-Carotene, Vitamin E and Selenium Levels Associated with Accelerated Carotid Atherogenesis in Hypercholesterolemic Finnish Men, *Circulation 87*, 2.
19. Bierenbaum, M.L., Reichstein, R.P., Bhagavan, H.N., and Watkins, T.R. (1992) Relationship Between Serum Lipid Peroxidation Products in Hypercholesterolemic Subjects and Vitamin E Status, *Biochem. Int. 28*, 57–66.
20. Qureshi, A.A., Qureshi, N., Hasler-Rapacz, J.O., Weber, F.E., Chaudhary, V., Crenshaw, T.D., Gapor, A., Ong, A.S.H., Chong, Y.H., Peterson, D., and Rapacz, J. (1991) Dietary Tocotrienols Reduce Concentrations of Plasma Cholesterol, Apolipoprotein B, Thromboxane B_2 and Platelet Factor 4 in Pigs with Inherited Hyperlipidemias, *Am. J. Clin. Nutr. 53*, 1042S–1049S.
21. Qureshi, A.A., Qureshi, N., Wright, J.J.K., Shen, Z., Kramer, G., Gapor, A., Chong, Y.H., Dewitt, G., Ong, A.S.H., Peterson, D.M., and Bradlow, B.A. (1991) Lowering of Serum Cholesterol in Hypercholesterolemic Humans by Palmvitee, *Am. J. Clin. Nutr. 53*, 1021S- 1026S.
22. Wahlquist, M., Krivokuca-Bogetic, Z., Lo, C.S., Hoge, B., Smith, R., and Lukito, W. (1992) Differential Serum Responses of Tocopherols and Tocotrienols During Vitamin Supplementation in Hypercholesterolemic Individuals Without Change in Coronary Risk Factors, *Nutr. Res. 12*, S181–S201.
23. Watkins, T.R., Lenz, P., Gapor, A., Struck, M., Tomeo, A., and Bierenbaum, M.L. (1993) γ-Tocotrienol as a Hypocholesterolemic and Antioxidant Agent in Rats Fed Atherogenic Diets, *Lipids 28*, 1113–1118.
24. Bierenbaum, M.L., Fleischman, A.I., Raichelson, R.I., Hayton, T., and Watson, P.B. (1973) Ten-Year Experience of Modified-Fat Diets on Younger Men with Coronary Heart Disease, *Lancet 2*, 1404–1407.
25. Miettinen, T.A., Huttunen, J.K., Naukkaunien, V., Strandberg, T., and Vanhanen, H. (1986) Long-Term Use of Probucol in the Multifactorial Primary Prevention of Vascular Disease, *Am. J. Card. 57*, 49H–54H.
26. Bocan, T.M.A., Mazur, M.J., Mueller, S.B., Charlton, G., Kieft, K.A., and Krause, B.R. (1994) Atherosclerotic Lesion Development in Hypercholesterolemic Japanese Quail Following Probucol Treatment: A Biochemical and Morphological Evaluation, *Pharmacol. Res. 29*, 65–76.
27. Johansson, J., Olsson, A., Bergstrand, L., Elinder, L.S., Nilsson, S., Erikson, U., Molgaard, J., Holme, I., and Waldius, G. (1995) Lowering of HDL_{2b} by Probucol Partly Explains the Failure of the Drug to Affect Femoral Atherosclerosis in Subjects with Hypercholesterolemia, *Arterio. Vasc. Biol. 15*, 1049–1056.
28. Atkins, D., Psaty, B.M., Koepsell, T.D., Longstreth, W.T., and Larson, E.B. (1993) Cholesterol Reduction and the Risk of Stroke in Men. A Meta-Analysis of Randomized, Controlled Trials, *Ann. Intern. Med. 119*, 136–145.
29. Herbert, P.R., Gaziano, J.M., and Hennekens, C.H. (1995) An Overview of Trials of Cholesterol Lowering and Risk of Stroke, *Arch. Int. Med. 155*, 50–55.

[Received November 5, 1993, and in final revised form October 31, 1995; Revision accepted October 31, 1995]

ANNOUNCEMENTS

CHANGE OF REFERENCE STYLE FOR *LIPIDS*

References should be used to quote published work as well as papers accepted for publication ("in press"). References are quoted in the text in numerical order (arabic numerals, in parentheses). **Titles of journal articles and chapter titles in multi-author books are to be included immediately preceding the name of journal or book.** When consecutive citations refer to the same journal, the source abbreviation should be given in each case (Avoid "*Ibid.*"). Each reference should refer only to one source. References are to be typed double-spaced on a separate page following the acknowledgments.

Literature citations *must* conform to the following format:

- For reference to journal articles:

  Turner, E.M., Miller, E., and Smith, D.E. (1975) Temperature and Diet Effects on Trout, *J. Biol. Chem. 250,* 108–115.

- For reference to books:

  Turner, E.H., and Smith, D.E. (1964) *Enzymes,* 2nd edn., pp. 565–567, Academic Press, New York.

- For reference to multi-author books:

  Turner, E.H. (1967) Temperature and Diet Effects on Trout, in *Comprehensive Biochemistry* (Florkin, E.M., and Stotz, E.M., eds.) Vol. 28, pp. 23–65, Elsevier, Amsterdam.

- For reference to theses:

  Turner, E.H. (1969) Fatty Acid Metabolism, Ph.D. Thesis, University of Wisconsin, Madison, pp. 12–14.

- Manuscripts accepted for publication should be listed as:

  Turner, E.H., Temperature and Diet Effects on Trout, *J. Biol. Chem.,* in press.

Names of journals must be abbreviated according to *Chemical Abstracts Service Source Index* (CASSI), published by Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210. These abbreviations are identical to those used in *Chemical Abstracts. These abbreviations are to be used exclusively.*

If it is necessary to refer to "unpublished data," "personal communication," "manuscript in preparation" or papers "submitted for publication," such citations should not be listed as references, but are to appear in the text, e.g., (Turner, E.H., and Smith, D.E., unpublished data). Authors must obtain written permission to cite the unpublished work of others, and copies of such correspondence must accompany the manuscript. Avoid the use of *et al.* in referring to authors: list all names.

NOTE: All titles in References section should be capitalized. See examples above.

NOTE: *Lipids* is now italicizing all mathematical variables. This may not have been clear in January 1995 "Guide to Authors."

AVAILABILITY OF PURIFIED OMEGA-3 FATTY ACIDS

Kilogram quantities of EPA and DHA ethyl ester capsules are available free of charge through the Fish Oil Test Materials Program, a joint effort of the National Oceanic and Atmospheric Administration and National Institutes of Health. These quality-assured and quality-controlled materials contain 94% EPA or 90% DHA. Ethyl esters of vegetable oils (olive, corn, and safflower) are available in capsule form as controls. Funded research projects studying the effect of

(continued on next page)

omega-3 fatty acids in health and disease, from all funding sources, are eligible to receive these capsules. For further information or to request a short application form, contact the Fish Oil Test Materials Program at 12501 Washington Ave., Rockville, MD 20852 (Phone: 301-443-9643; Fax: 301-594-0035).

CALENDAR

February 1996
Fourth International Symposium on Hyphenated Techniques in Chromatography (HTC 4), Feb. 6–9, 1996, Saint John's Conference Center, Bruges, Belgium. Five workshops (hands-on training as well as tutorials) will be held Feb. 4–5. (1) Large volume injection: the next step in capillary gas chromatography for trace analysis; (2) Pre- and postcolumn techniques in HPLC for improved analyte isolation, derivatization, cleanup, separation, and detection; (3) Isotopically labeled compounds in hyphenated GC techniques; (4) Analytical tools for GC–MS (advanced modes of ion-trap spectrometry); (5) Biomedical applications of GC–MS. Information: Dr. Robert Smits, Congress Secretariat, Ordibo bvba, L. Henninckstraat, B-2610 Wilrijk (Antwerpen), Belgium [Phone/Fax: 32 (3)828.89.61].

May 1996
XVIth Washington International Spring Symposium on Frontiers in Bioactive Lipids—'96, May 6–9, 1996, Washington, D.C., USA. Information: Dr. Jack Y. Vanderhoek, Chair—Symposium '96, Dept. of Biochemistry and Molecular Biology, The George Washington University, 2300 Eye St., N.W. RN 5530, Washington DC 20037-2337 USA (Phone: 202-994-2929; Fax: 202-994-8974; E-mail: jyvdh@gwis2.circ.gwu.edu.). Deadline for submission of abstracts: February 1, 1996.

July 1996
European Thrombosis Research Organisation and the International Society for the Study of Fatty Acids and Lipids in cooperation with Summer University of Maastricht, July 10–12, 1996, University of Maastricht, The Netherlands, presents "Lipids, Membranes and Thrombosis (fundamental basis of cardiovascular disease and its prevention)." Information: Gerard Hornstra, Dept. of Human Biology, P.O. Box 616, 6200MD Maastricht, The Netherlands (Phone: +31-43-88 16 25; Fax: +31-43-67 09 76; E-mail: G. Hornstra @ HB.RuLimburg.NL).

July–August 1997
Fourth International Congress on Essential Fatty Acids and Eicosanoids, July 27–August 1, 1997, Edinburgh, Scotland. Information: Secretariat, c/o Meeting Makers, 50 George St., Glasgow G1 1QE, Scotland, United Kingdom.